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Hot Water-Soluble Glycosides: Location in the Tissue of *Populus grandidentata* Bark

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HOT WATER-SOLUBLE GLYCOSIDES: LOCATION IN THE TISSUE OF *POPULUS GRANDIDENTATA* BARK

A thesis submitted by

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TABLE OF CONTENTS

SUMMARY

The bark of a Populus grandidentata Michx. tree felled in May was found to be rich in extractive components. Extraction with various organic solvents and hot water removed 25% of the original solids. The remaining bark was composed of about 56% holocellulose (35% alpha-cellulose) and about 19% of material insoluble in 72% sulfuric acid.

Separation of this bark into distinct anatomical layers [phellem (3%), phelloderm (17%), and inner portion (22%) and outer portion (58%) of the secondary phloem] was accomplished after a brief steaming period. Fractionation of the ethyl acetate-soluble hot water extractives from each layer and from the whole unsteamed bark by elution polyamide column chromatography yielded nine crystalline glucosides. Two of these glucosides, which have been designated as grandidentoside and populoside, were isolated and identified during the present investigation. Elution with water yielded salicin, salicortin, 1-O-p-coumaroyl-β-D-glucose, populin, tremuloidin, grandidentatin, grandidentoside, and salireposide. Continued elution with an aqueous ethanol gradient gave populoside. This is the first time that salicortin has been reported in the bark of P. grandidentata. Pyrocatechol and salicyl alcohol were also identified. Glucose, fructose, sucrose, and maltose were found in the hot water raffinate after extraction with ethyl acetate; glucose, fructose, galactose, xylose, mannose, and arabinose were present after hydrolysis. Maltose has been reported only occasionally as being present in plants.

Except for salicortin, all components isolated from the tissue layers also were obtained from the ethyl acetate extractives of the whole unsteamed bark. Salicortin was isolated in low yield from the outer portion of the secondary phloem; only salicyl alcohol and populin were identified in the phellem.

Salicin was the primary glucoside found during this investigation and amounted to about 9 g. per 1500 g. of ovendry bark solids. The amount of 1-O-p-coumaroyl-β-D-glucose (3 g.) also was found to be quite significant. More than 20 g. of crystalline material were isolated and identified from 132 g. of the whole bark ethyl acetate-soluble portion from 230 g. of hot water extractives. In all cases, the yields of crystalline components from the individual tissue layers were lower than from the whole unsteamed bark extractives. A total crystalline yield from all layers amounted to 9.4 g. Specific analysis for the quantity of 1-O-p-coumaroyl-β-D-glucose indicated that only 40% of the total amount present in the whole bark could be accounted for in the tissue layers.

Treatment of the ethyl acetate extracts with lead subacetate liberated essentially all of the tremuloidin, whereas only traces were obtained without treatment. Lead subacetate treatment eliminated salicortin, grandidentoside, and pyrocatechol from the clarified extract, and increased the salicin quantity slightly. Decreased amounts of 1-O-p-coumaroyl-β-D-glucose, grandidentatin, and salireposide were observed.

The structure of populoside was determined by mass spectrometry, infrared absorption spectroscopy, alkaline hydrolysis, enzymatic hydrolysis, and ultimate analysis to be the ω-caffeic acid ester of salicin [o-(3,4-dihydroxycinnamoyloxy-methyl)phenyl β-D-glucopyranoside].

Crystalline grandidentoside always was isolated in conjunction with grandidentatin. Separation was achieved by silica gel column chromatography using a methanol-chloroform gradient elution technique. Grandidentoside was found to be extremely sensitive and a trace of acid was necessary to inhibit

degradation when working with this substance. By mass spectrometry, infrared absorption spectroscopy, and alkaline hydrolysis, its structure was shown to be a caffeic acid ester of cis-2-hydroxycyclohexyl β-D-glucopyranoside. Mass spectrometry and nuclear magnetic resonance spectrometry strongly suggested substitution at the C-2 position of the glucose moiety.

Nuclear magnetic resonance spectrometry has proven to be a useful analytical tool in determining substitution of complex phenolic glucosides, especially where only small quantities of material are available and the molecule does not lend itself to standard procedures of identification.

Polyamide column chromatography afforded a major separation of the ethyl acetate extractive mixture. However, the eluate fractions were still quite complex. Silica gel column chromatography was shown to be suitable for further fractionation of these components.

INTRODUCTION

Bark residues have created a problem in the wood industry for many years. Barking involves expenditure of both capital and time, and the waste collected results in disposal difficulties. Since this component amounts to between 8 and 15% of the total weight of the tree (1), a significant percentage of the basic raw material is being discarded. Normally used as fuel, bark wastes constitute "potential raw material sources of tremendous magnitude" (2). To tap this potential for effective utilization, an understanding of the complex chemical and morphological composition of bark is necessary. However, an equally important value of such information is its relationship to the biochemistry and physiology of the plant and its consequent use for the cultural and genetic improvement of a species (3). Such knowledge can be achieved only through concerted research in the field of bark chemistry.

What is true for barks in general holds for the bark of aspen. The use of this species for pulpwood in the Lake States has increased annually from less than 100,000 cords in 1936 and 1,000,000 cords in 1956, to over 2,198,000 cords in 1964 (1,4). As the volume of aspen used increases, so does the volume of bark unused.

During the past several years, extensive work has been done in the area of bark hot water extractives from the family Salicaceae (5-21). A brief review (22) indicates that activity in the area of processing extractives from the leaves and barks of various species is increasing at a rather fast pace. Much of the work is being directed toward the isolation of pharmaceutically useful components.

Past research in all areas of bark chemistry has placed emphasis on using the whole bark as the basic starting material. Relatively little attention

has been given to the connection between the anatomy of the bark and its chemical composition (23). Certain elements of the bark serve as avenues of translocation, storage areas for food reserves, and protective sheaths for the stem. Therefore, one might expect the extractives of different regions to vary both in nature and in concentration (3). An analysis of the hot water extractives has not been approached beginning with the anatomical tissue layers rather than with the whole bark. Consequently, it was the purpose of this work to determine whether or not separation of a bark into its anatomical subfractions would be an improvement over utilization of the whole bark as a starting material in detecting the presence of components in the hot water extractives. The premises were that new constituents which were diluted by the whole bark could be isolated and the assignment of components to specific regions of the bark would have some chemical-biological significance for future work.

Populus grandidentata, bigtooth aspen, was chosen for study since (a) it is an important pulpwood species, (b) it could be separated easily into various tissue layers, (c) it is rich in extractives, and (d) newer methods of fractionation and chromatographic procedures never had been applied to the bark of this tree.

The methods and results of studies on the chemical composition of a variety of barks have been summarized in reviews by Segall and Purves (24) and Kurth (25). Although not present in these reviews, approximate analyses for whole aspen bark were given in later reports by Browning and Bublitz (26) and Chang and Mitchell (27). Hossfeld and Kaufert (1) have shown that P. tremuloides can be separated into four distinct tissue layers using a steaming and hand fractionation procedure. Hossfeld and Hunter (3) followed up this investigation with a more thorough examination of the petroleum ether extractives.

Aspen bark was found to be rich in extractive material. Successive extractions with organic solvents and water removed as much as 36% of the original bark (1).

Although early work on the barks of several Populus species by Pearl, et al. (28) involved an alkaline hydrolysis treatment, characterization of the hot water extractives became more important in later studies. The hot water extracts of P. grandidentata bark and other important species were clarified by lead subacetate treatment and analyzed for glycosides and phenolic compounds (5). Salicin* was found in all barks studied and salireposide in all but one. Tremuloidin was found only in the barks of P. grandidentata and P. tremuloides. A qualitative investigation of bigtooth aspen bark indicated the presence of salicin, tremuloidin, salireposide, salicyl alcohol, p-coumaric acid, vanillic acid, and gentisyl alcohol. In a continuing study of the barks of the family Salicaceae, the hot water extract of the bark of a P. grandidentata tree felled in September was studied (7). The clarified extract yielded, in addition to the components listed above, populin, ferulic acid, p-hydroxybenzoic acid, sucrose, glucose, and fructose. Grandidentatin was identified in a sample of July bark (6).

For the removal of interfering materials, previous investigators used primarily magnesium oxide and lead subacetate (29). A disadvantage of this treatment was that it removed indefinite amounts of phenolic glycosides by precipitation (29) and destroyed or altered the nature of certain glycosides (8, 14). Separation of the hot water extractives of the bark was believed to be best accomplished by preliminary extraction with ethyl acetate followed by elution polyamide column chromatography (15, 18, 29-31). Since these techniques are performed under neutral conditions, only a minimal destruction of the original components would be expected.

*For structures refer to Fig. 4 and 5, pages 23 and 24.

In this investigation, P. grandidentata bark was separated into four anatomical layers. Each layer, in addition to the whole unsteamed bark, was extracted with hot water. The ethyl acetate-soluble portion of the hot water extractives was further fractionated by elution polyamide column chromatography with water and then with a gradient elution technique using aqueous ethanol.

PRESENTATION AND DISCUSSION OF RESULTS

Aspen bark is morphologically composed of three well-defined layers (1, 3, 32). After steaming under pressure, the smooth green bark of Populus grandidentata can be separated with greater ease into distinct tissue layers. The first (outer) layer corresponds morphologically with the phellem (Fig. 1) and makes up 3% by weight of the dry bark. The second layer is a soft green mass of slightly tangentially elongated parenchyma cells and amounts to 17% of the dry weight of the bark. This layer is considered to include the phelloderm and cortex and may contain stone cells accompanied by small amounts of fiber. The predominant layer is the secondary phloem which may be subdivided arbitrarily into two regions. Fibers intermixed with stone cells make up the outer portion (Layer 3): fibers, sieve tubes, ray cells, and other parenchyma cells are combined to form the inner layer (Layer 4). Layer 3 comprises 58% of the bark; Layer 4 makes up 22%. The stone cells of P. grandidentata and P. tremuloides, according to Haas and Kremers (33), are analytically similar to the wood of the same species.

The data accumulated during different fractionations indicated that steaming and hand isolation permitted separation of the whole bark into specific layers. A small amount of material (1.2% based on the ovendry weight of bark) was lost during steaming and carried away with the condensate.

ANALYSIS OF POPULUS GRANDIDENTATA BARK

Few investigators have approached the problem of relating chemical composition to the anatomy of the bark. Consequently, prior to the specific discussion of the hot water extractives from P. grandidentata bark and their fractionation, some attention was devoted to a general analytical characterization. This established a more fundamental background for subsequent work.

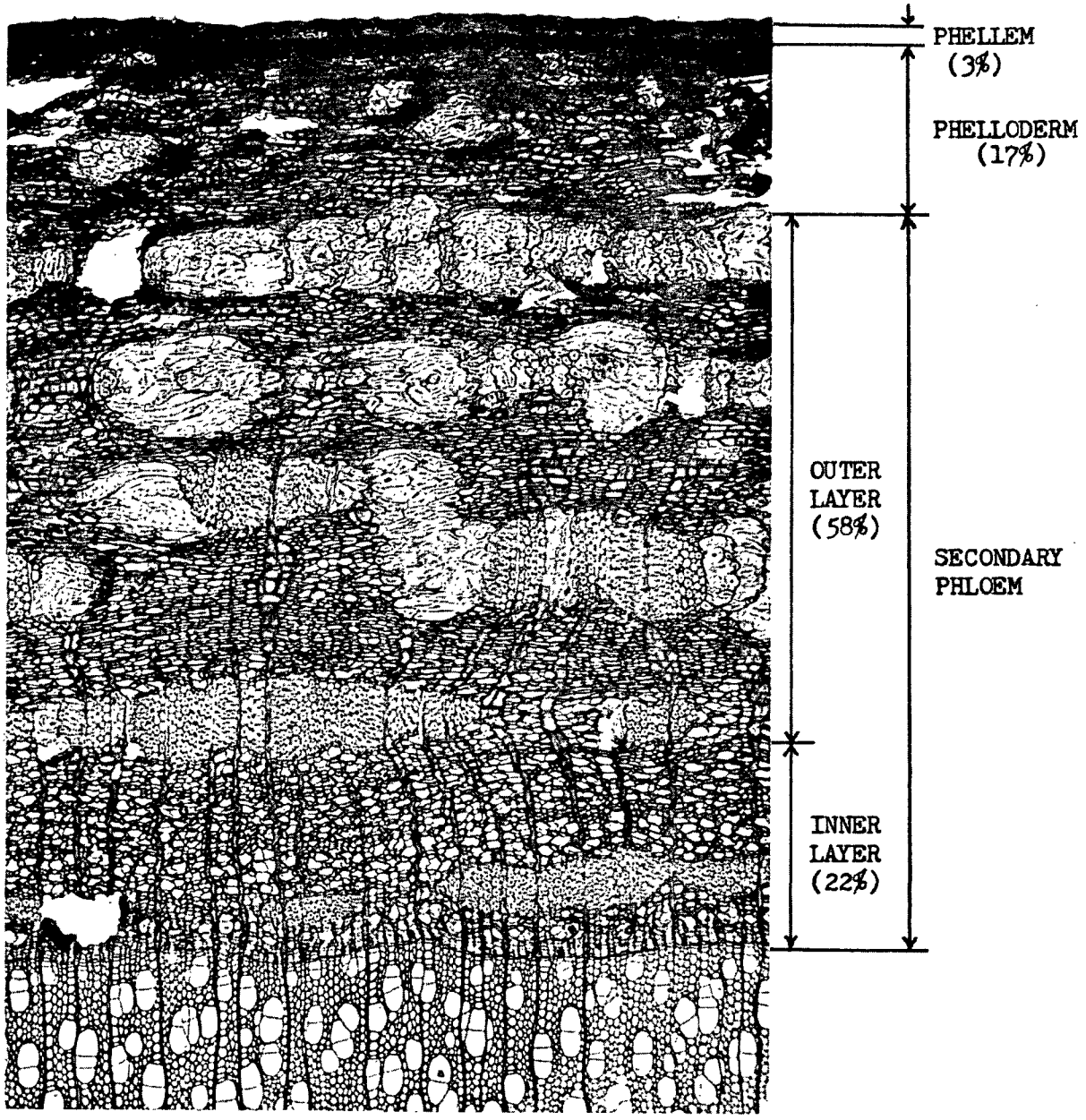

Figure 1. Cross Section of Populus grandidentata Bark (55X)

DISTRIBUTION OF EXTRACTIVES IN *POPULUS GRANDIDENTATA* BARK

The various tissue layers and the whole bark were classified according to the nature of the extractives present. Using the general scheme of Hossfeld and Kaufert (1), duplicate samples were exhaustively extracted with petroleum ether, ether, benzene, ethanol, and hot water. Analytical results may be found in Table I.

Extraction with the above solvents removed about 26% of the original bark. It is interesting to note the distribution of these extractives within the bark. Based on the whole bark, the major proportion of extractives were found in the outer region of the secondary phloem. The highest concentration of extractives was found in the phellem (51%). Although the phelloderm exhibited a high concentration of extractives (34%), this concentration is considerably lower than that reported for *P. tremuloides* (77%). In all cases the ethanol and hot water extractives constitute a significant portion of the total solids removed. Any difference between the results of the present work and that for *P. tremuloides* may be accounted for by (a) difference in species, (b) age of the trees, or (c) time of the year when the trees were cut.

A sample of the whole bark had not been steamed; it was used as a control in this study. A comparison between the extractive data for the whole unsteamed bark and for the tissue layers (Table II) illustrated that steaming did modify the nature of the extractives.

TABLE I

DISTRIBUTION OF EXTRACTIVES IN POPULUS GRANDIDENTATA BARK

	Tissue Elements							
	Phellem Layer 1 (3%)		Phelloderm Layer 2 (17%)		Secondary Phloem Layer 3 (58%)		Secondary Phloem Layer 4 (22%)	
	Layer[a], %	Bark[b], %	Layer, %	Bark, %	Layer, %	Bark, %	Layer, %	Bark, %
Petroleum ether	11.80	0.35	6.88	1.20	4.14	2.39	8.54	1.88
Ether	4.01	0.12	1.71	0.29	0.81	0.47	0.84	0.18
Benzene	2.10	0.06	0.91	0.16	0.40	0.23	0.47	0.10
Ethanol	27.70	0.80	18.14	3.10	12.14	7.04	13.89	3.05
Hot water	5.03	0.15	6.45	1.13	5.28	3.06	6.04	1.33
Total	50.64	1.48	34.09	5.88	22.77	13.19	29.78	6.54

[a]Based on ovendry weight of layer.

[b]Based on ovendry weight of original whole bark.

TABLE II

COMPARISON OF STEAMED AND UNSTEAMED BARK EXTRACTION DATA

	Extractives	
	Whole Bark Unsteamed Yield, %	Tissue Layers[a] Yield, %
Petroleum ether	6.59	5.82
Ether	5.22	1.06
Benzene	0.45	0.55
Ethanol	8.55	13.99
Hot water	3.91	5.67
Total	24.72	27.09

[a]Based on the extractive weights summed from each tissue layer.

The ether extractives of the various tissue layers were found to be considerably lower in yield than those of the whole unsteamed bark; the ethanol and hot water extractives of the separate tissue fractions were present in greater amounts than the whole bark extractives. In each case the total extractive content found was nearly the same. Possible explanations for this effect were (a) degradation of components during steaming and fractionation and/or (b) modification of the physical structure of the bark which might alter the accessibility of solvents to the extractives.

ANALYSIS OF EXTRACTIVE-FREE POPULUS GRANDIDENTATA BARK

Since the extractives of P. grandidentata bark amounted to only 26% of the total weight, an analysis of the remaining components was made. Table III lists the alpha-cellulose, holocellulose, ash, material insoluble in 72% sulfuric acid, and methoxyl content for the whole bark and each tissue layer. These data are an average of two tests.

Jensen, et al. (23) indicated that conventional methods for the quantitative determination of lignin are totally unsatisfactory when applied to bark. For this reason the phrase "material insoluble in 72% sulfuric acid," as defined in the Klason lignin analysis, was used in place of the term "lignin." Apparently, cork or suberin appears as lignin if not removed prior to analysis. Carbohydrate determinations, however, can be accomplished by the same type of methods which are applied to wood.

Wangerin (34) reported the following data for P. tremuloides bark: holocellulose, 60.6%; Cross and Bevan cellulose, 38.7%; lignin, 23.9%; and ash, 4.1%. His data correspond very closely with those found in the present study.

The methoxyl content of the material insoluble in 72% sulfuric acid was 21.5% in the inner bark (Layer 4), which corresponds with the methoxyl content of hardwood lignin (23). Analysis progressing toward the outer layers indicated that the methoxyl content dropped sharply.

Table IV contains a summative analysis for the whole bark and each tissue layer. Only Layer 1 exceeded 100% to any degree. A high value was reasonable, since this layer for smooth green bark is the protective sheath and likely would contain a significant amount of suberin. Because the suberin was not removed beforehand, it overlapped into more than one analysis.

TABLE III

DISTRIBUTION OF COMPONENTS IN EXTRACTIVE FREE POPULUS GRANDIDENTATA BARK

Tissue Layer	Holocellulose[c], %	Alpha-cellulose, %	Ash, %	Material[c] Insoluble in 72% H_2SO_4, %	Methoxyl[d], %
Layer 1[a]	84.4	40.9	5.5	73.6	3.8
Layer 2[a]	76.4	48.8	5.1	25.9	14.6
Layer 3[a]	80.7	53.2	2.8	30.9	15.6
Layer 4[a]	81.4	53.8	2.7	16.5	21.5
Whole bark[b]	80.5	50.4	4.0	25.1	16.4

[a]Based on ovendry weight of extractive-free layer.

[b]Based on ovendry weight of extractive-free original whole bark.

[c]Suberin has not been removed and accounts for summative yields over 100%.

[d]Based on the weight of material insoluble in 72% H_2SO_4.

TABLE IV

SUMMATIVE ANALYSIS FOR POPULUS GRANDIDENTATA BARK[a]

Tissue Layer	Holocellulose Yield, %	Material Insoluble in 72% H_2SO_4 Yield, %	Extractives Yield, %	Total Yield, %
Whole Bark	60.6	18.9	24.7	104.2
Layer 1	45.0	39.3	46.6	130.9
Layer 2	50.4	17.1	34.0	101.5
Layer 3	62.1	23.8	22.8	108.7
Layer 4	57.0	11.6	29.8	98.4

[a]Percentages based on the individual ovendry tissue weights.

SOLUBLE SUGAR ANALYSIS

An analysis of the sugars present in the hot water extracts of the tissue layers and in the whole bark showed that glucose and fructose were present in all samples; sucrose and maltose were only in the whole bark and in Layers 3 and 4. After hydrolysis, glucose, fructose, galactose, and arabinose were detected in each of the layers; mannose and xylose were detected in the whole bark and in Layers 2 and 3. The presence of galactose, mannose, arabinose, and xylose after hydrolysis suggested that they exist as water-soluble hemicelluloses. Maltose was observed only within the inner layers of the bark. Only occasionally has maltose been reported to exist *per se* in plants. However, since it is a product of the enzymatic hydrolysis of starch and since both amylases and starch are found in the inner bark of many species, it may be a degradation product (35, 36).

ANALYSIS OF POPULUS GRANDIDENTATA BARK HOT WATER EXTRACTIVES

The primary goal of this thesis was to characterize the hot water extractives from P. grandidentata bark as a function of the anatomical tissue layers, placing specific emphasis on the phenolic glycosides present. Ethyl acetate extraction of the hot water extracts was used to remove the phenolic components and to leave the carbohydrate fraction in the aqueous phase. Further fractionation of the ethyl acetate extractives was accomplished by elution polyamide column chromatography with water and then with a gradient elution technique using aqueous ethanol.

HOT WATER EXTRACTION OF POPULUS GRANDIDENTATA BARK

All finely divided layers were extracted batchwise with boiling water. Duplicate hot water extractions were performed on each of the tissue layers; a single sample of the whole unsteamed bark was extracted as a control. The ovendry weight of each layer extracted was based on a whole bark sample size of 1500 g. and on the relative size of each layer. Data for one series of extractions may be found in Table V; data for the second series are located in Table XXIII (Appendix I). Except for the whole bark, each sample listed in Table V was extracted three times; those in Table XXIII were extracted four times.

The colors of the hot water extracts varied. Extracts of Layers 1 and 3 were a very rich chocolate brown; that of Layer 2 was somewhat lighter in appearance; that of Layer 4 was almost a tan color.

Although Layer 3 contained most of the hot water extractives, Layer 2 exhibited the highest concentration (19.6%). Since the extractives removed by hot water amounted to about 14% of the dry bark, they constituted a significant

TABLE V

DISTRIBUTION OF HOT WATER EXTRACTIVES IN POPULUS GRANDIDENTATA BARK

Tissue Layer	Weight of Tissue[b] Layer, g.	Hot Water Extractives Yield, g.	Tissue Layer Yield, %	Whole Bark Yield, %
Layer 1[a]	45	3.94	8.75	0.26
Layer 2[a]	275	52.4	19.1	3.50
Layer 3[a]	855	94.6	11.1	6.30
Layer 4[a]	325	48.0	14.8	3.20
Total	1500	198.9		13.26
Whole bark[c]	1500	230.3		15.35

[a]Based on three batch hot water extractions.

[b]Based on the contribution of each layer to the ovendry weight of 1500 g. whole bark solids.

[c]Based on four batch hot water extractions.

portion of the total extractive content of the bark (26%). The figure of 14% compares closely with the amount of hot water extractives removed from the green barks of P. trichocarpa (16%) (12) and P. balsamifera (14%) (14).

FRACTIONS FROM LAYER 3

An interesting phenomenon was observed when Layer 3 was extracted with hot water. After being stirred in water for a period of time, this layer separated into essentially two fractions if the agitation were discontinued. A heavier fraction, probably stone cells and sclereidlike material, settled to the bottom; a lighter fraction remained suspended in the aqueous phase. The two fractions were separated. After freeze drying, the lighter, suspended material was a powdery substance amounting to roughly 10% of Layer 3. Observation under a microscope using various staining techniques led to the conclusion that this powdery substance consisted of sieve tube elements or other parenchyma cells because they were rich in extractives. Further investigation showed that more than 80% of the hot water extractives came from the lighter fraction.

ETHYL ACETATE EXTRACTION OF THE HOT WATER EXTRACTIVES

The concentrated hot water extracts were extracted exhaustively with ethyl acetate. Extractive data in Table VI (solvent free) correspond with the hot water extractive data in Table V; those in Table XXIV (Appendix I) (residual solvent present) correspond with the hot water extractive data in Table XXIII (Appendix I). Again Layer 2 was found to be the richest in extractives. The ethyl acetate extractives amounted to between 35 and 40% of the total extractive content of the bark.

The yield of ethyl acetate extractives based on the ovendry weight of bark was under 10%, somewhat lower than that observed for the smooth green bark

TABLE VI

DISTRIBUTION OF ETHYL ACETATE SOLUBLE-HOT WATER EXTRACTIVES IN POPULUS GRANDIDENTATA BARK

Tissue Layer	Period of Extraction, hr.	Ethyl Acetate[a] Extractives Yield, g.	Hot Water Extract Yield, g.	Tissue Layer Yield, %	Whole Bark Yield, %
Layer 1[b]	350	1.31	33.2	2.91	0.09
Layer 2[b]	360	30.3	57.9	11.0	2.02
Layer 3[b]	360	57.0	60.3	6.68	3.80
Layer 4[b]	290	17.6	36.7	5.41	1.17
Total		106.2			7.08
Whole bark[c]	570	130.8	56.8		8.72
Condensate	430	5.85	57.6		0.39

[a]Extractives dried on polyamide to remove solvent.

[b]Based on first series of three batch hot water extractions.

[c]Based on second series of four batch hot water extractions.

of P. balsamifera (10.1%) (21) and considerably lower than the yield obtained for triploid P. tremuloides (20.0%) (15).

POLYAMIDE COLUMN CHROMATOGRAPHY OF THE ETHYL ACETATE EXTRACTIVES

Elution chromatography on a polyamide column was used to further fractionate the complex ethyl acetate-soluble components of the hot water extractives. Ethyl acetate extractives from the following sources were fractionated: the whole bark, Layer 1, Layer 2, Layer 3, Layer 4, the whole bark extractives after treatment with lead subacetate, and the condensate. Elution was carried out using water and, in some instances, continued using a step-gradient technique, going from water to 95% ethanol. Fractions were collected and monitored by means of thin-layer chromatography*. Weights of all fractions were noted, and elution curves were obtained.

Chromatography of the various ethyl acetate fractions on a polyamide column led to the isolation of several glucosides, two of which had not previously been identified. Populoside (I), (Fig. 2) isolated during gradient elution chromatography, is the ω-caffeic acid ester of salicin. Grandidentoside (II), isolated during elution with water, is cis-2-hydroxycylohexyl 2-O-caffeoyl-β-D-glucopyranoside and is one of two possible diastereoisomers.

Known crystalline glucosides were identified by thin-layer and paper chromatography, melting points, mixed melting points, and in certain instances, infrared spectroscopy.

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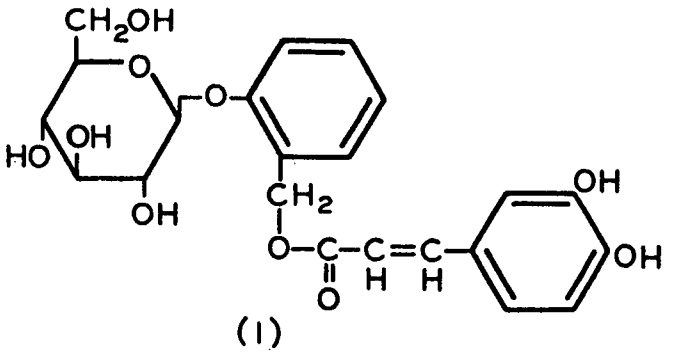
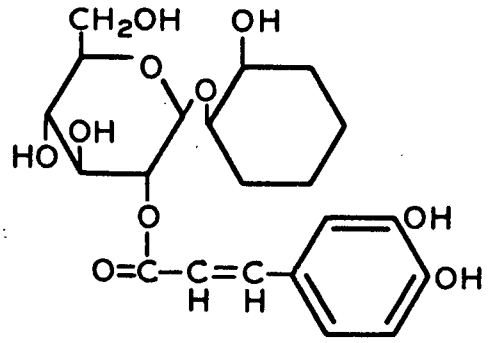
*Thin-layer chromatography – unless otherwise specified, this term refers to silica gel plates developed in 4:1 chloroform-methanol (trace of acetic acid), sprayed with sulfuric acid, and heated in an oven at 105°C. for 10 min.



(I)



(II)

Figure 2. Structures of Populoside (I) and Grandidentoside* (II)

Whole Bark

The elution chromatogram for the whole unsteamed bark ethyl acetate extractives is presented in Fig. 3. Elution of the whole bark extractives will be considered in detail because it is typical of the remaining tissue layers.

Depicted below the elution curve is the thin-layer chromatography data accumulated during polyamide fractionation. The components in each fraction are illustrated by plotting the fraction number against the R_f of each component

*One of two possible diastereoisomers.

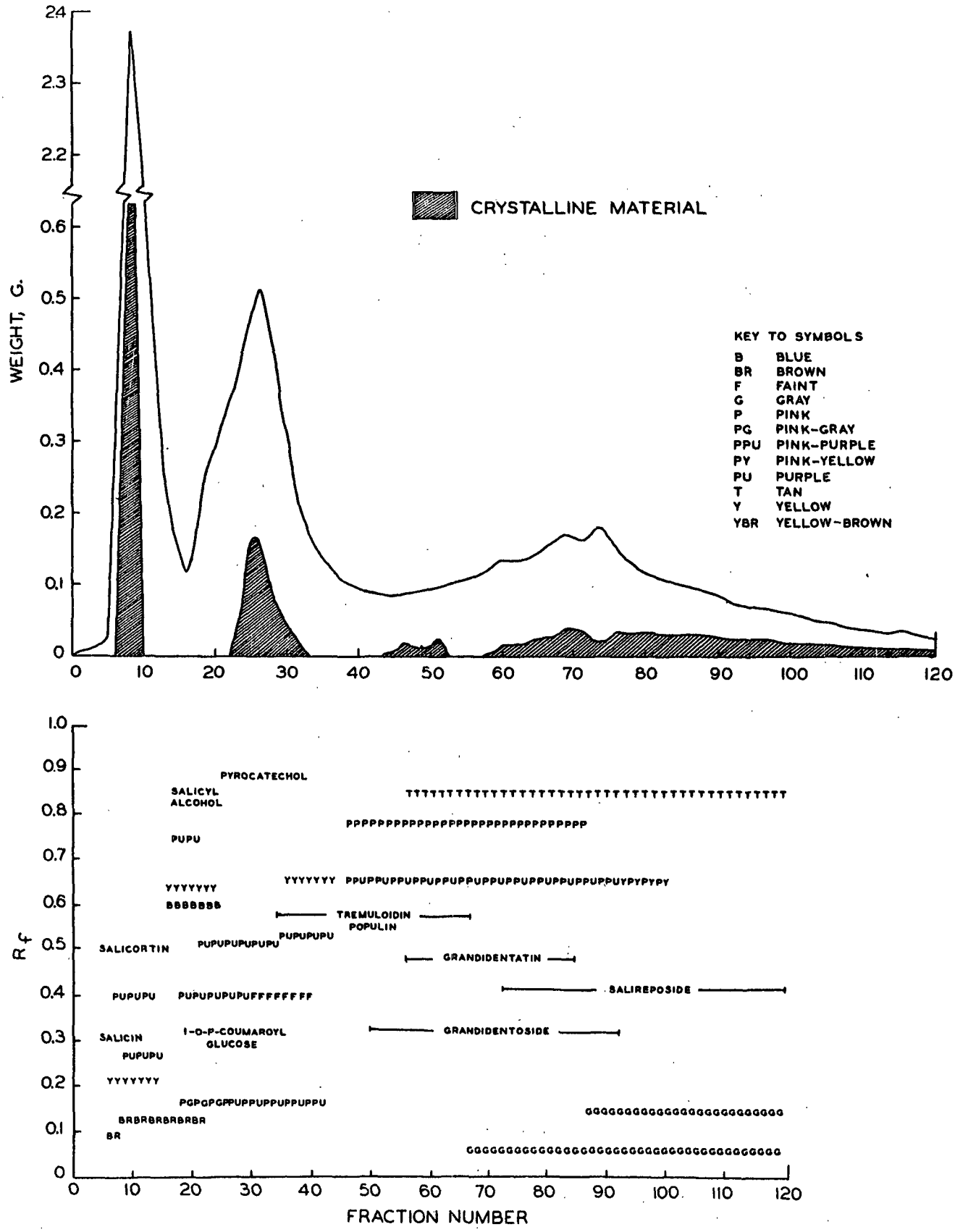


Figure 3. Polyamide Column Chromatography of the Ethyl Acetate Extractives from Populus grandidentata Whole Bark-2 and Associated Thin-Layer Chromatography Data. Weight of Extractives Applied to the Column: 35.4 g.

using symbols to represent colors. Known compounds were listed by name in the appropriate location. The figure shows the complexity of the extractive mixture and indicates that no fraction is chromatographically pure. Though a major separation was achieved, overlapping of components was a common occurrence.

Duplicate ethyl acetate extractive samples from the whole bark were fractionated on polyamide in an attempt to determine the reproducibility. The first sample analyzed was designated as whole bark-1; the second was designated as whole bark-2. The latter sample, discussed at this time, came from the same stock of ethyl acetate extractives but was fractionated about nine months after the first sample. The elution curve for the first fractionation may be found in Fig. 26 (Appendix II).

Concentration of eluate fractions 7-9 gave a high yield of salicin (III) (Fig. 4) crystals. After the salicin fractions, a significant amount of 1-O-p-coumaroyl-β-D-glucose (IV) was deposited by Fractions 23-32. Upon concentration, Fractions 44-51 crystallized to yield a small amount of populin (V) (Fig. 5) and possibly some tremuloidin (VI). Crystalline fractions from 58 to 120 were usually a combination of grandidentatin (VII), grandidentoside (II), and salireposide (VIII). Grandidentatin and grandidentoside were found in about equal concentration from Fraction 58 to 79. Grandidentoside and salireposide were found in admixture until Fraction 90. Only pure salireposide was found from Fraction 90 to 120.

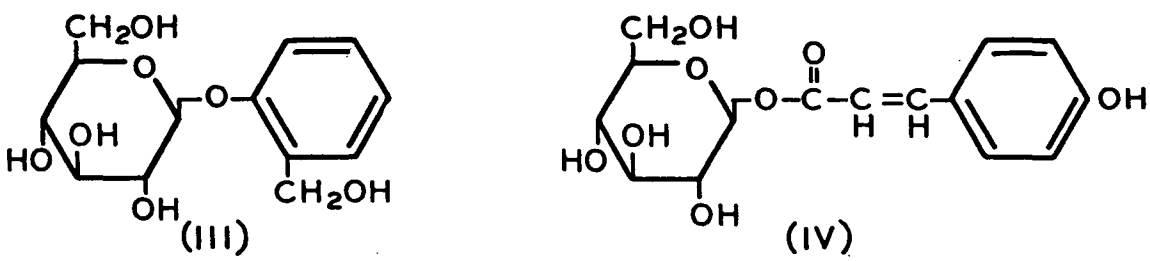
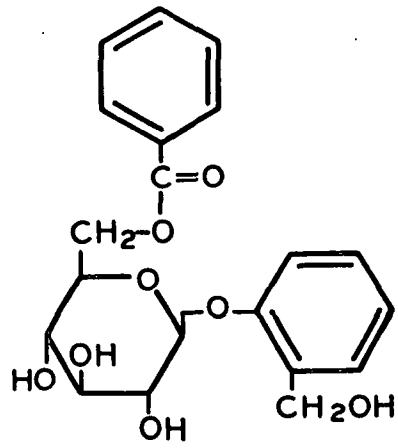
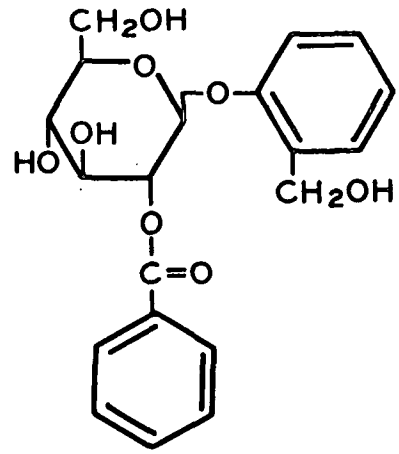


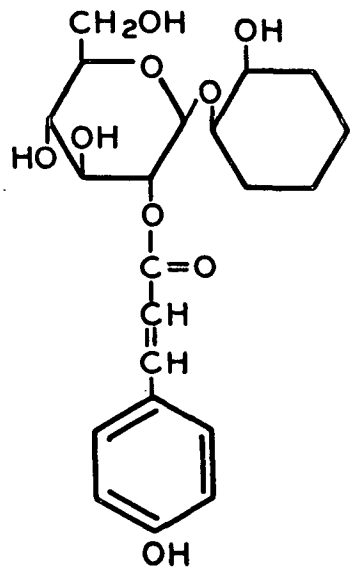
Figure 4. Structures of Salicin (III) and 1-O-p-Coumaroyl-β-D-glucose (IV)



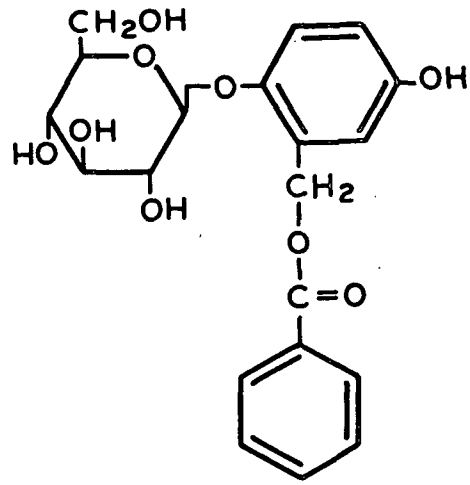
(V)



(VI)



(VII)



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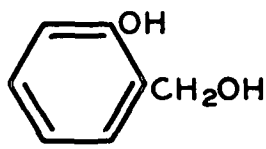
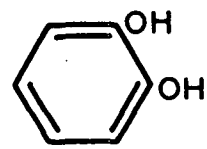


(IX)



(X)

Figure 5. Structures of Populin (V); Tremuloidin (VI); Grandidentatin (VII); Salireposide (VIII); Salicyl Alcohol (IX); and Pyrocatechol (X).

In addition to the abovementioned crystalline components, salicyl alcohol (IX), Fractions 17-22, and pyrocatechol (X), Fractions 25-37, were detected by thin-layer and quantitatively determined by gas chromatography. Quantitative data for all of the components are summarized in Table VIII.

Of the 35.4 g. of ethyl acetate extractives placed on the column, 21.9 g. (62%) were eluted after 120 fractions (24 liters of eluate). A rather significant portion of the extractives (24%) was eluted in the first few fractions. Following water elution, the remaining extractives were removed from the polyamide by extraction with hot water and with hot ethanol. An overall material balance indicated that about 11% of the extractives remained associated with the polyamide. A complete material balance is given in Table XII.

Fractionation of two equivalent samples of whole bark ethyl acetate extractives on a polyamide column exhibited similar elution curves as illustrated in Fig. 3 and Fig. 26 (Appendix II, page 104). In each case the same crystalline components, except for populin and tremuloidin, were identified. The primary differences observed were (a) larger quantities of extractives in the first few fractions, (b) higher yields of crystalline components, and (c) a sharper elution curve. The higher crystalline yield was attributed to improved experimental techniques while the sharper elution curve was probably a result of a continuous rather than intermittent elution.

Layer 1

Figure 6 illustrates the water-eluted polyamide chromatogram of the ethyl acetate extractives from Layer 1. Since only 1.31 g. of extractives were available a much smaller polyamide column was used, and smaller volumes of eluate were collected in each fraction.

Crystals were found only in Fractions 33-43. These crystals exhibited a pink spot on thin-layer, melted at 178-9°C., and did not depress a mixed melting point with pure populin. Salicyl alcohol was detected in Fractions 6-15 by thin-layer chromatography and comprised a major portion of the peak in that region.

Layers 2, 3, and 4

Layers 2, 3, and 4 had similarly shaped elution curves; each exhibited three primary peaks (Fig. 7, 8, and 9). Only the relative quantity of material eluted varied.

Salicin was the primary crystalline component in each layer and was found under the first major peak. Salicortin, a glucoside of undetermined structure first reported by Thieme (29, 30, 37-39), was isolated in fractions immediately after salicin in Layer 3. It has been shown to yield salicyloyl salicin on hydrolysis (39). Thieme believes that salicortin is a precursor of salicin in most species and breaks down while the bark extractives are being prepared for analysis. During the course of this work, salicortin was known by thin-layer chromatography to be present, but it could not be isolated in crystalline form. Salicortin was recently crystallized from a dry sirup in absolute ethanol (40). This technique was applied to previously fractionated extractives, but salicortin could be crystallized only from Layer 3. Although found in small yield, salicortin has been shown to exist in P. grandidentata bark. Thin-layer chromatography indicated that salicortin was absent or of minimal concentration in Layers 1 and 2, existing in greater concentrations in Layers 3 and 4.

1-O-p-Coumaroyl-β-D-glucose was isolated in each of the tissue layers between Fractions 25 and 40. Small amounts of tremuloidin were observed in Layers 3 and 4 in the region of Fractions 50-60. Crystalline grandidentatin

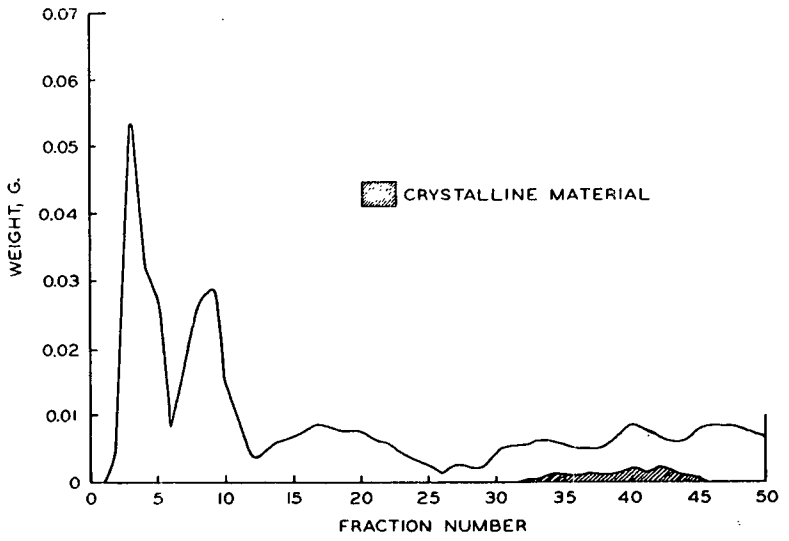


Figure 6. Polyamide Column Chromatography of the Ethyl Acetate Extractives from Populus grandidentata Bark-Layer 1. Weight of Extractives Applied to the Column: 1.31 g.

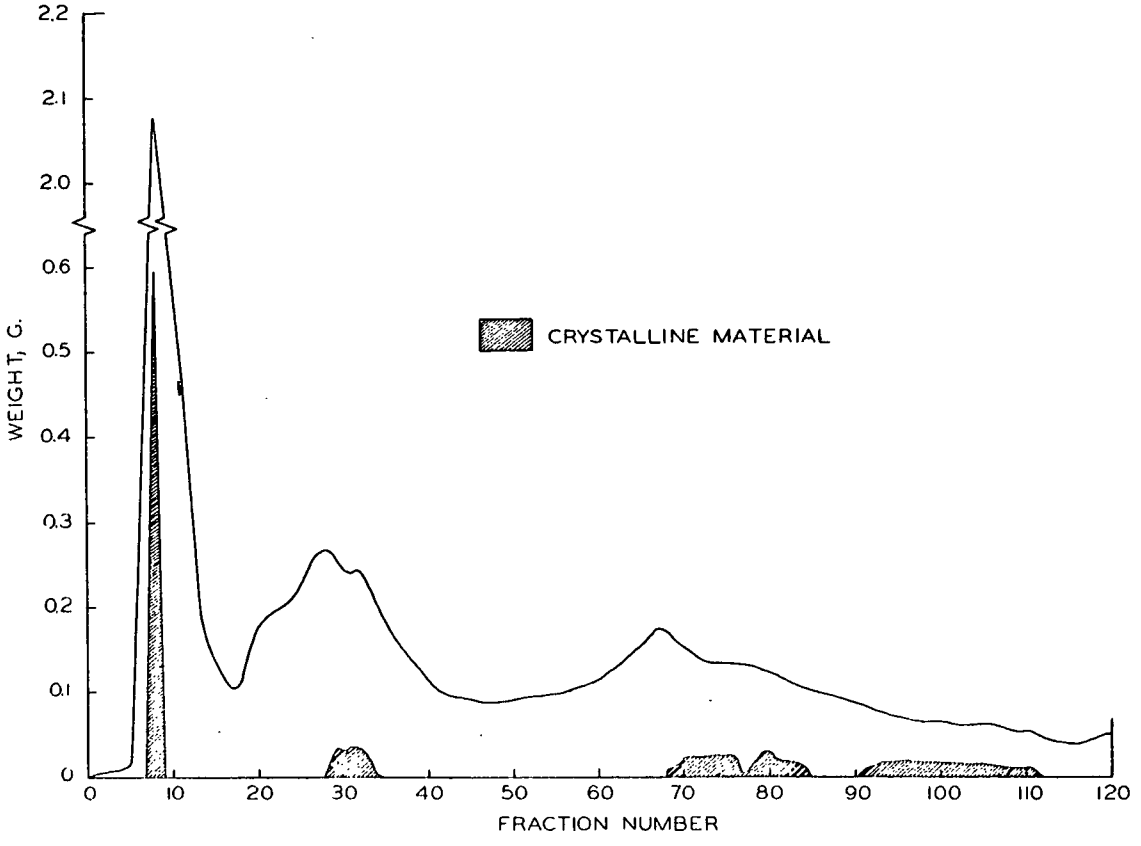


Figure 7. Polyamide Column Chromatography of the Ethyl Acetate Extractives from Populus grandidentata Bark-Layer 2. Weight of Extractives Applied to the Column: 30.3 g.

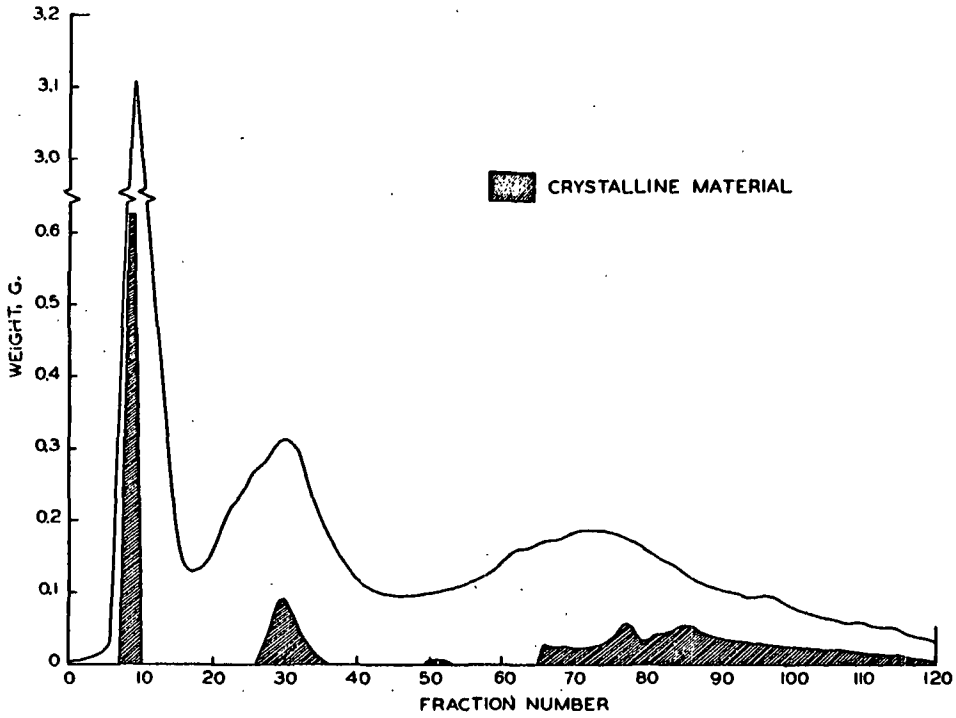


Figure 8. Polyamide Column Chromatography of the Ethyl Acetate Extractives from Populus grandidentata Bark-Layer 3. Weight of Extractives Applied to the Column: 36.4 g.

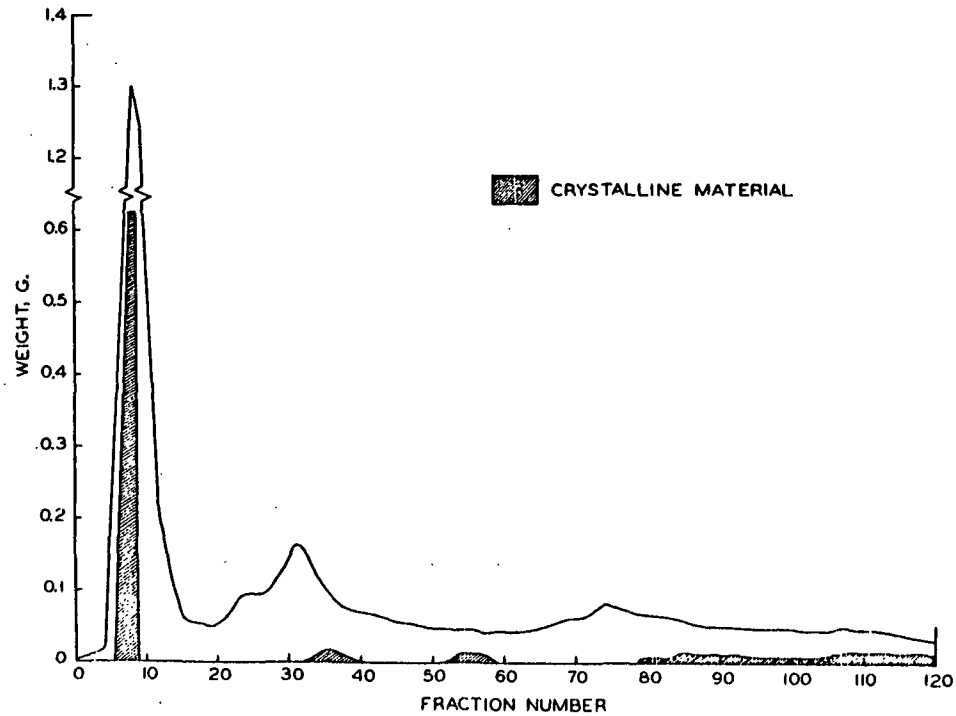


Figure 9. Polyamide Column Chromatography of the Ethyl Acetate Extractives from Populus grandidentata Bark-Layer 4. Weight of Extractives Applied to the Column: 17.6 g.

and grandidentoside again were found in combination in all three layers between Fractions 65 and 90. From Fractions 80 to 90 salireposide was isolated in combination with grandidentatin and/or grandidentoside; in later fractions it usually was quite pure.

Although some salicyl alcohol was detected in Layer 4, only traces were noted in Layers 2 and 3. Pyrocatechol was identified in each of the three layers.

Over 50 individual components were detected by thin-layer chromatography in the ethyl acetate extractives of Layers 2 and 4 eluted by water from the polyamide columns; 35 components were detected in Layer 3.

Of the extractives placed on the columns for each of the layers, 26, 30, and 30% were eluted in the initial fractions of Layers 2, 3, and 4, respectively. In each case, this was a rather significant portion of the extractives.

Crystalline components are summarized in Table VII and complete material balances are given in Table XII.

Condensate

The "condensate" was that water which condensed while the bolts of wood were steamed and was collected as the effluent from the steam trap. This water may have condensed on the bark removing extractives in the process. An elution chromatogram on polyamide of the ethyl acetate extractives from this fraction showed one large peak in the early region of the elution, indicating the presence of primarily water-soluble components (Fig. 10). On thin-layer no spots were observed which corresponded with any of the major components found in the tissue layers. In the intermediate fractions some salicyl alcohol was noticed, but its concentration was negligible.

TABLE VII

IDENTIFIED COMPONENTS FROM THE POLYAMIDE FRACTIONATION OF THE ETHYL[a] ACETATE SOLUBLE-HOT WATER EXTRACTIVES FROM POPULUS GRANDIDENTATA BARK TISSUE LAYERS

Component	Layer 1 Yield, g.	Layer 2 Yield, g.	Layer 3 Yield, g.	Layer 4 Yield, g.	Total Yield, g.
Salicortin	–	–	P	P	–
Salicin	P	0.60	1.78	1.18	3.56
1-O-p-Coumaroyl-β-D-glucose	–	0.16	0.70	0.08	0.94
Grandidentatin	–	P	P	P	–
Grandidentoside	–	P	P	P	–
Combined grandidentatin and grandidentoside	–	0.31	1.74[b]	0.15	2.20
Salireposide	–	0.31	0.87	0.38	1.56
Populin	0.02	–	–	–	0.02
Tremuloidin	–	–	0.02	0.06	0.08
Salicyl alcohol	0.05	T	T	0.02	0.07
Pyrocatechol	T	0.13	0.46	0.30	0.89
Populoside	–	–	0.36	–	0.36
Total	0.07	1.51	5.93	1.90	9.41

[a] Based on a summative tissue weight of 1500 g. o.d. bark solids (Table V).

[b] Crude crystals from Fractions 66 to 96. Includes grandidentatin, grandidentoside, and salireposide, among other components.

P = Present in quantity, but not determined.

T = Trace of material present.

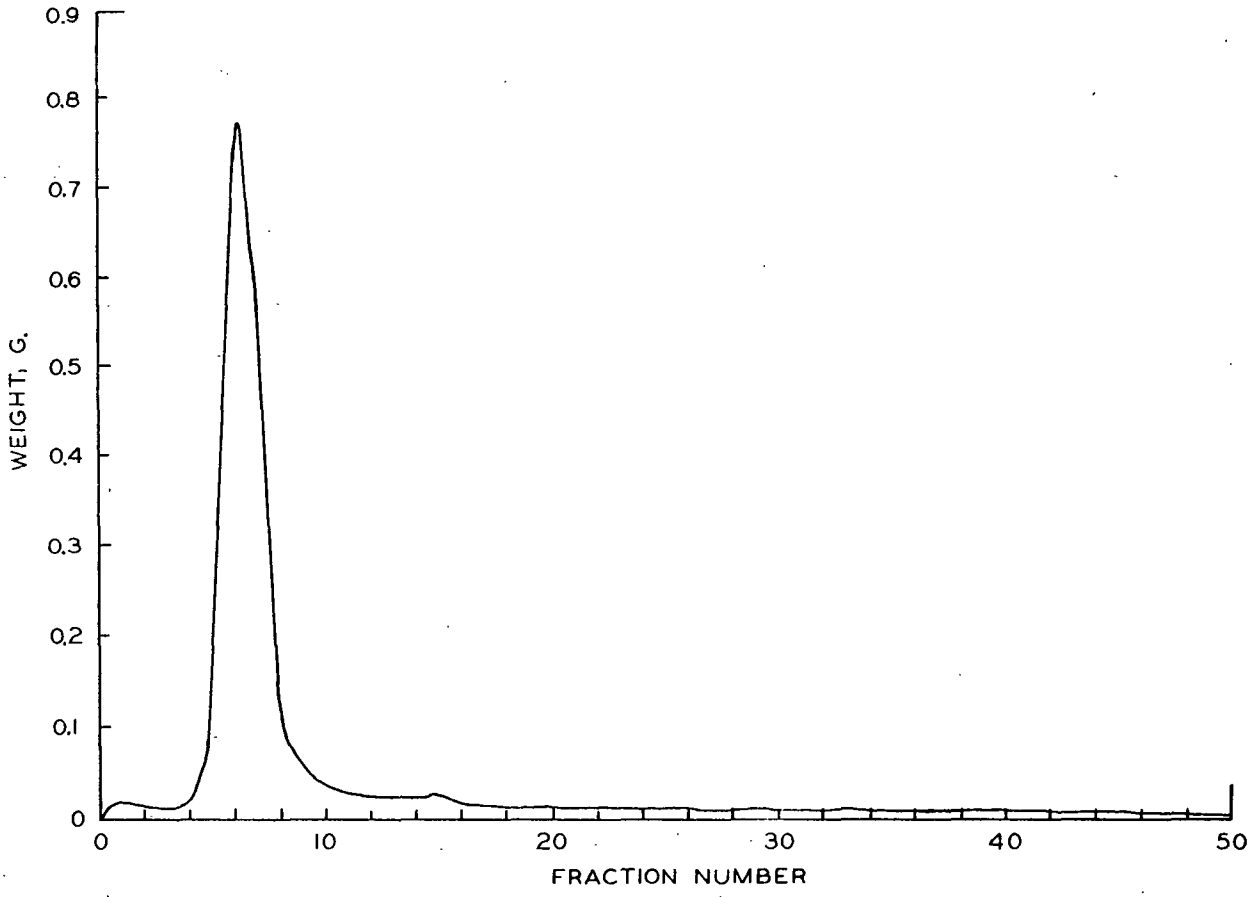


Figure 10. Polyamide Column Chromatography of the Ethyl Acetate Extractives from the Condensate Recovered During the Steaming of Populus grandidentata Bark. Weight of Extractives Applied to the Column: 2.93 g.

Gradient Elution Polyamide Column Chromatography

After water elution of the ethyl acetate extractives on a polyamide column, a rather significant proportion of the extractives (30-40%) still remained. In the past (15, 31), standard practice has been to extract the polyamide with hot water and with hot ethanol to remove the extractives. Possibly the full benefit of the column was not being utilized and fractionation of the residual components could be achieved by changing the solvent or solvent mixture. Since most of the extractives remaining on the column were more soluble in ethanol, a solvent gradient going from water to ethanol appeared to be most favorable.

Initially, the step-gradient approach was applied directly to the extractives from Layers 2 and 3 remaining on the column after elution with water. Elution chromatograms from this work indicated that most of the extractives were eluted in one large peak (Fig. 27 and 28 - Appendix II) due to the steep gradient applied. Consequently, the Layer 3 ethyl acetate extractives were rechromatographed on a polyamide column. This time a much shallower gradient was induced and as Fig. 11 shows, a greater separation of components was achieved. Using this technique, 230 mg. of a new glucoside, populoside, were isolated and identified. No special methods were required to obtain crystalline populoside which crystallized merely upon concentration of the fractions between 80 and 90.

In addition to populoside, other crystalline components were obtained, but in much lower yields. Fraction 7 gave a few mg. of salicin, and some crystalline salireposide was observed between Fractions 55 and 60. The crystals between Fractions 70 and 75 were quite impure, containing some sirupy material. On thin-layer the primary constituent exhibited a yellow spot with a blue-purple center. Concentration of fractions between 117 and 134 produced a waxy or amorphous material.

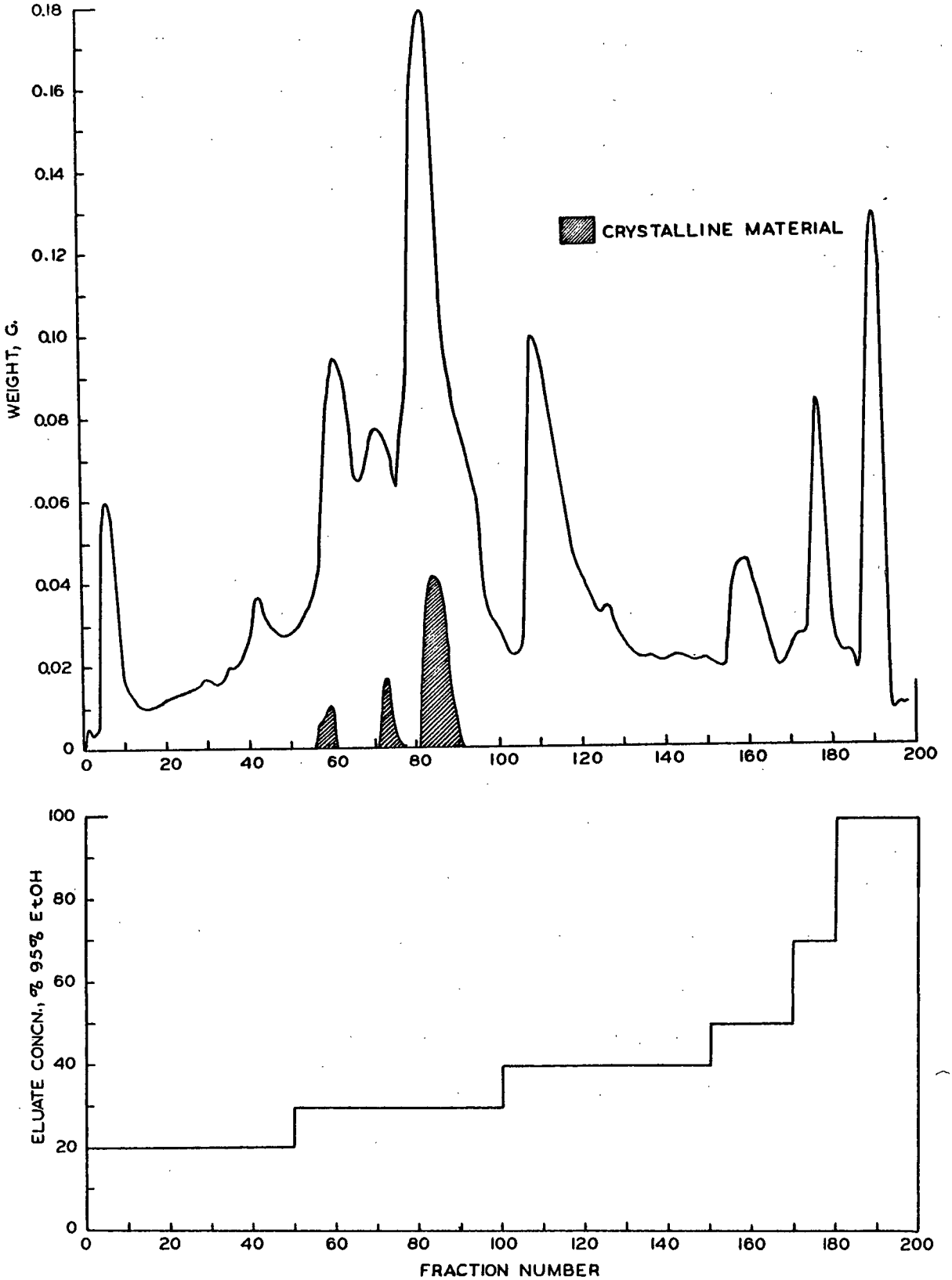


Figure 11. Gradient Elution Polyamide Column Chromatography of the Ethyl Acetate Extractives from Populus grandidentata Bark-Layer 3. Weight of Extractives Applied to the Column: 8.78 g.

Application of this latter gradient elution technique to the whole bark-2 ethyl acetate extractives after elution with water yielded populoside in greater concentration (Fig. 12). Populoside amounted to about 1.5 g. per 1500 g. of ovendry bark. No other crystalline components were observed.

Concentration of most fractions in both gradient elutions produced rich golden sirups rather than crystalline fractions. Thin-layer chromatography indicated primarily gray or brown spots when sprayed with sulfuric acid and heated. A large number of compounds exhibited no colored spot on thin-layer but were visible when observed under ultraviolet light.

LEAD SUBACETATE CLARIFICATION OF THE WHOLE BARK ETHYL ACETATE EXTRACTIVES

Previous work (5, 7) involving lead subacetate clarification of P. grandidentata bark hot water extractives indicated that tremuloidin was a prominent component of this species. Work on the nonlead subacetate-treated extractives in this thesis demonstrated that neither tremuloidin nor populin* exist in appreciable quantity in P. grandidentata bark.

Whole bark ethyl acetate extractives were submitted to a mild lead subacetate treatment. After removal of the precipitated lead salts, the filtrate was freed of lead by precipitation with hydrogen sulfide and filtration. Of the ethyl acetate extractives originally treated with lead subacetate, only 37% remained soluble and were recovered in the regenerated filtrate. The clarified filtrate was concentrated, placed on a polyamide column, and subjected to elution chromatography as discussed previously.

*Populin can be formed by acyl migration of the benzoyl group in tremuloidin.

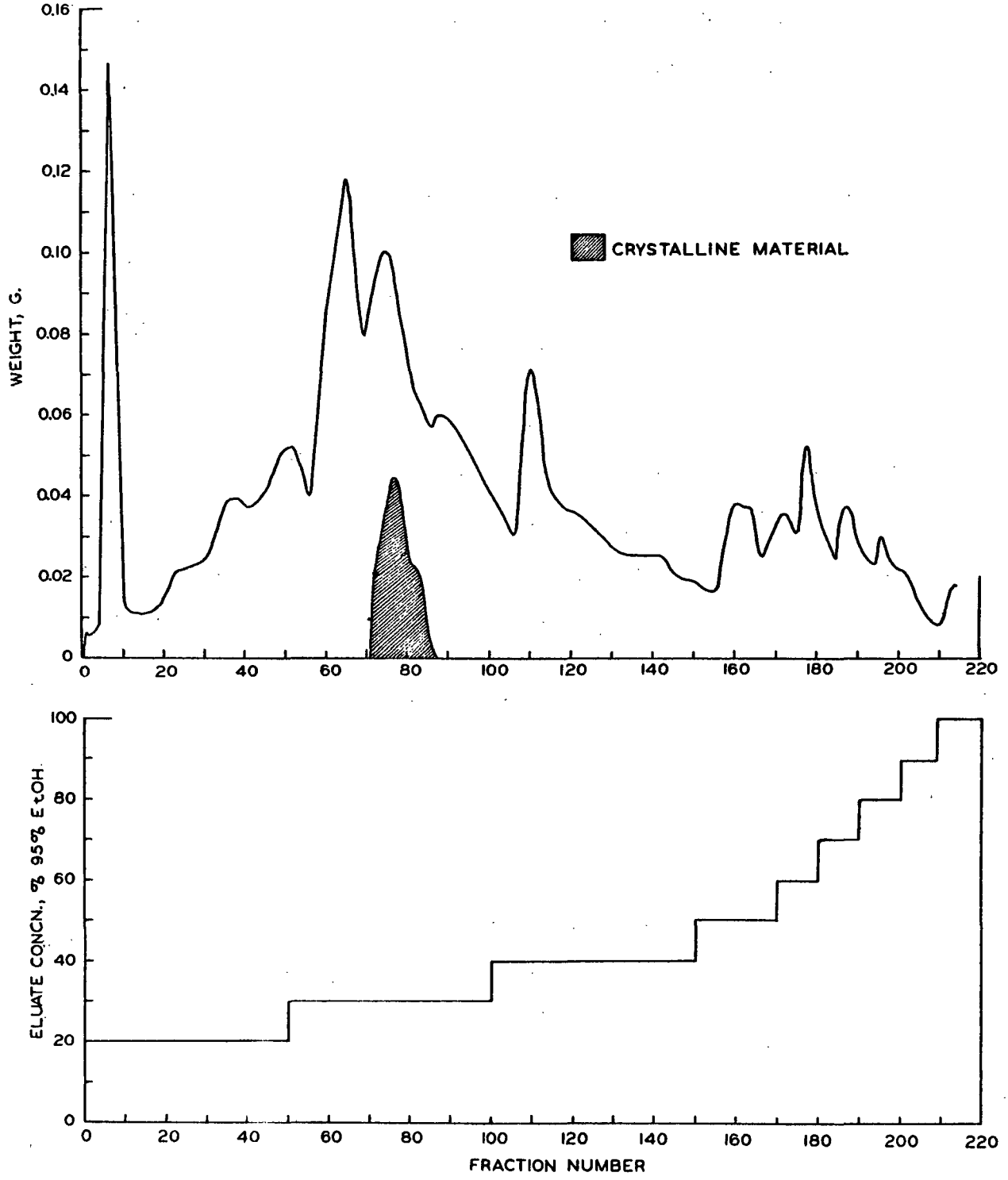


Figure 12. Gradient Elution Polyamide Column Chromatography of the Ethyl Acetate Extractives from Populus grandidentata Whole Bark. Weight of Extractives Applied to the Column: 13.4 g.

An elution chromatogram of the clarified ethyl acetate extractives is given in Fig. 13. Although salicin and 1-O-p-coumaroyl-β-D-glucose were again the principal crystalline components observed in the early portion of the chromatogram, 0.825 g. of tremuloidin was recovered between Fractions 48 and 67.

No tremuloidin was found in the first elution of the whole bark extractives; some was found in Layers 3 and 4, and slightly larger amounts were noted in the second elution of the whole bark extractives. The presence of tremuloidin in the latter elutions probably resulted from the degradation of a precursor with time.

Treatment with lead subacetate caused other changes: (a) an increased yield of salicin was observed; (b) decreased yields of 1-O-p-coumaroyl-β-D-glucose, grandidentatin, and salireposide were noticed; and (c) the absence of salicortin, grandidentoside, and pyrocatechol was indicated by thin-layer chromatography.

Pyrocatechol and salireposide were probably removed as the lead salts (14, 41). Grandidentoside may have been precipitated as the lead salt or degraded since it is extremely sensitive to alkali. Salicortin, the possible precursor of salicin, most likely was converted into salicin or other degradation products.

Of the extractives placed on the polyamide column, an extremely large portion (47%) was eluted in the first few fractions. Complete material balances for both crystalline components and extractives are given in Tables VIII and XII, respectively.

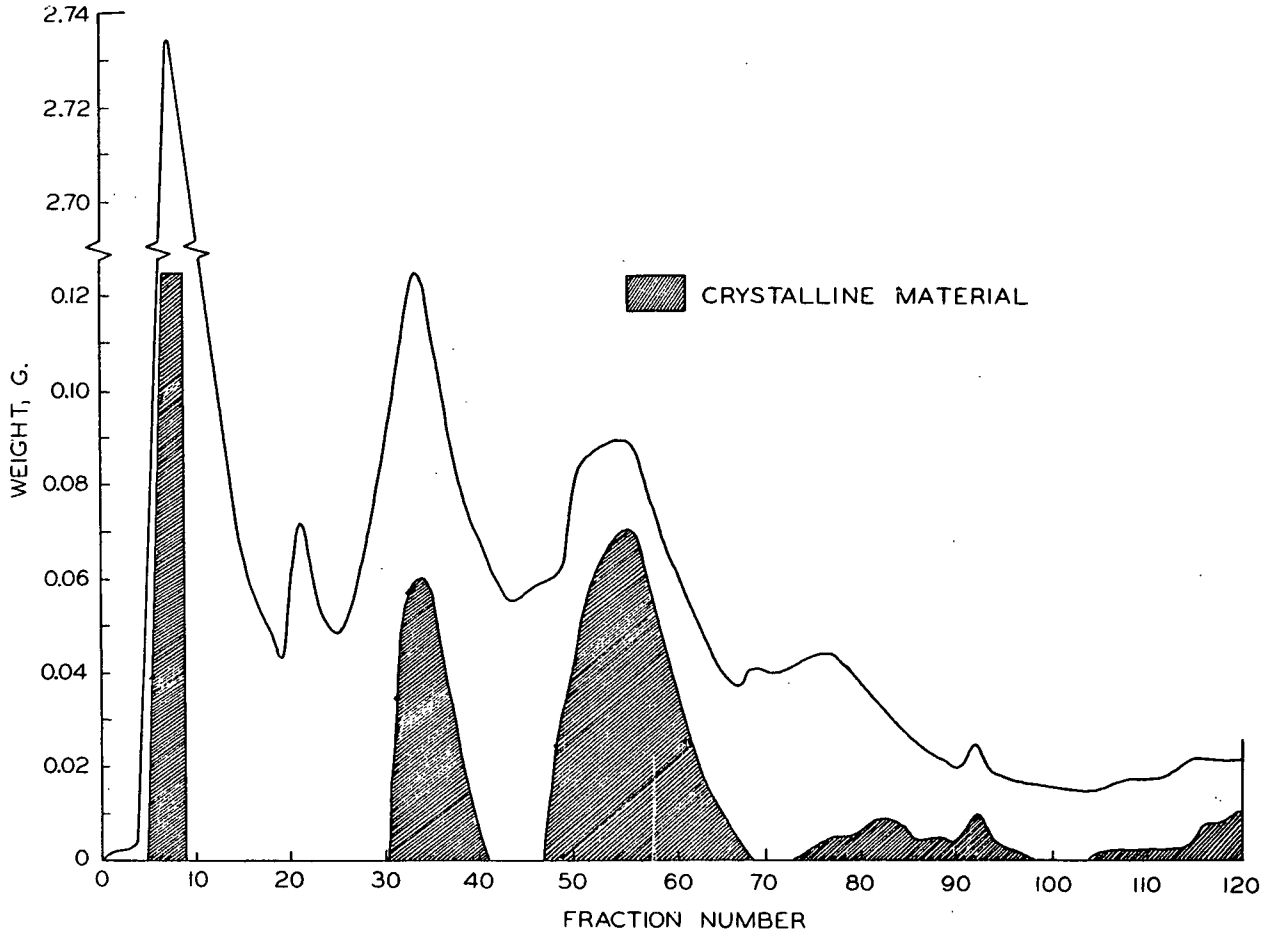


Figure 13. Polyamide Column Chromatography of the Ethyl Acetate Extractives from Populus grandidentata Whole Bark After Treatment with Lead Subacetate. Weight of Extractives Applied to the Column: 16.2 g.

TABLE VIII

IDENTIFIED COMPONENTS FROM THE POLYAMIDE FRACTIONATION OF THE ETHYL[a] ACETATE SOLUBLE-HOT WATER EXTRACTIVES FROM POPULUS GRANDIDENTATA WHOLE BARK

Component	Whole Bark-1 Yield, g.	Whole Bark-2 Yield, g.	Whole Bark after Treatment with Lead Subacetate Yield, g.
Salicortin	P	P	A
Salicin	5.27	8.85	9.21
1-O-p-Coumaroyl-β-D-glucose	2.53	3.05	1.06
Grandidentatin	P	P	0.36
Grandidentoside	P	P	A
Combined grandidentatin and grandidentoside	0.70	1.79	–
Salireposide	1.21	1.81	0.73
Combined grandidentatin and salireposide	0.09	–	–
Combined grandidentoside and salireposide	–	1.17	–
Populin	–	0.35	0.08
Tremuloidin	–	–	2.50
Salicyl alcohol	0.07	0.07	P
Pyrocatechol	1.65	1.50	A
Populoside	–	1.53	–
Total	11.52	20.12	13.94

[a]Based on 1500 g. of original o.d. bark solids.

P = Present in quantity, but not determined.

A = Components not detected by thin-layer chromatography.

IDENTIFIED COMPONENTS

When water and aqueous ethanol elutions were employed, large-scale polyamide column chromatography fractionated the ethyl acetate soluble-hot water extractives from P. grandidentata bark so several crystalline components could be isolated. Not all fractions yielded crystals when concentrated; many produced only sirups. Thin-layer chromatography indicated a large number of components remaining in solution. Alternate chromatographic procedures are required to accomplish a further separation.

Tables VII and VIII present a complete summary of the crystalline and noncrystalline components isolated and identified in this study and their relative contribution to the bark fractions. The contributions of salicin and 1-O-p-coumaroyl-β-D-glucose are listed in Tables IX and X, respectively. Values in Tables VII and VIII, excluding salicyl alcohol and pyrocatechol, are indicative only of the weight of material that crystallized from solution; they do not represent the total value of a component present. In some instances the values reported may constitute a significant portion of the total components; in others they may be rather insignificant. Although in the past there has not been a suitable method for determining the total amount of phenolic glucosides, gas chromatographic techniques developed by Bolan and Steele (42) should permit future investigators to determine total, rather than merely crystalline, quantities. The crystalline yield depends upon the nature of the species as well as upon the experimental techniques employed.

Salicin is shown in Table VII to be the principal glucoside isolated in this study. It amounted to 0.59% in the whole bark-2 sample. 1-O-p-Coumaroyl-β-D-glucose (0.20%) was the next most significant component, followed by salireposide, populoside (0.10%), pyrocatechol (0.10%), grandidentoside, grandidentatin, and

TABLE IX

RECOVERY OF CRYSTALLINE SALICIN

Tissue Layer	Crystalline Yield, g.	% of Tissue Layer	% of Whole Bark
Layer 1[a]	–	–	–
Layer 2[a]	0.60	0.22	0.04
Layer 3[a]	1.78	0.21	0.12
Layer 4[a]	1.18	0.36	0.08
Total	3.56		0.24
Whole bark-1[b]	5.27		0.35
Whole bark-2[b]	8.85		0.59
Whole bark after[b] treatment with lead subacetate	9.21		0.61

[a]Based on a summative tissue weight of 1500 g. o.d. bark solids.
[b]Based on 1500 g. of original o.d. bark solids.

salicyl alcohol. More than 70% of the salicyl alcohol was found in the outer tissue fraction (Layer 1). Even though minor amounts of tremuloidin were observed in most of the layers, tremuloidin was evident primarily after treatment with lead subacetate. Salicortin also was found in rather small yields.

Polyamide column fractionation of the whole bark-1 ethyl acetate extractives yielded 2.53 g. of crystalline 1-O-p-coumaroyl-β-D-glucose per 1500 g. of ovendry bark (Table X). In comparison, only 0.94 g. was found by adding the weights in each tissue layer. It was not known whether this lower value was due

to the degradation of 1-O-p-coumaroyl-β-D-glucose or if more material remained in the fraction solutions from polyamide chromatography and had not crystallized.

TABLE X

RECOVERY OF CRYSTALLINE 1-O-p-COUMAROYL-β-D-GLUCOSE

Tissue Layer	Crystalline Yield, g.	% of Tissue Layer	% of Whole Bark
Layer 1[a]	–	–	–
Layer 2[a]	0.16	0.06	0.01
Layer 3[a]	0.70	0.08	0.05
Layer 4[a]	0.08	0.03	0.005
Total	0.94		0.07
Whole bark-1[b]	2.53		0.17
Whole bark-2[b]	3.05		0.20
Whole bark after[b] treatment with lead subacetate	1.06		0.07

[a]Based on a summative tissue weight of 1500 g. o.d. bark solids.
[b]Based on 1500 g. of original o.d. bark solids.

The concentration of residual 1-O-p-coumaroyl-β-D-glucose in the mother liquors of the crystalline fractions was determined by saponification of the compound and measurement of the liberated p-coumaric acid quantitatively by gas chromatography. Paper chromatograms of the eluate fractions indicated that no other p-coumaroyl derivatives were present in the saponified samples. Table XI contains a summary of the total and crystalline yields of 1-O-p-coumaroyl-β-D-glucose.

TABLE XI

RECOVERY OF 1-O-p-COUMAROYL-β-D-GLUCOSE

Tissue Layer	Total Yield, g.	Amount of Crystalline Material Recovered, g.
Layer 1[a]	–	–
Layer 2[a]	0.48	0.16
Layer 3[a]	1.27	0.70
Layer 4[a]	0.24	0.08
Total	1.99	0.94
Whole bark-1[b]	4.72	2.51

[a]Based on a summative tissue weight of 1500 g. of o.d. bark solids.

[b]Based on 1500 g. of original o.d. bark solids.

These data imply that the difference in yields between the tissue layers and the whole bark was due to the degradation of 1-O-p-coumaroyl-β-D-glucose. It also indicates that only about 50% of the material could be accounted for as a crystalline product. Degradation of this and other components may have resulted from (a) the high temperatures employed during steaming which would accelerate hydrolysis and/or (b) oxidation as the tissue layers were exposed to the air. The outer tissue layers after steaming had characteristic dark colors when compared with samples that had been freeze dried. If the whole bark was fractionated directly from the tree, all tissue layers became extremely dark.

EXTRACTIVE MATERIAL BALANCE

Table XII presents an extractive material balance for all polyamide column chromatography. Itemized are the solids eluted by water; eluted during gradient elution with aqueous ethanol, if applicable; and removed from the polyamide by extraction with hot water and hot ethanol.

The extractives eluted by water in 120 fractions from the various tissue layers and from the whole bark varied from 60 to 69%, the average being 65%. During gradient elution, another 24 to 28% of the ethyl acetate extractives were eluted from the polyamide columns. Most of the extractives were eluted with 95% ethanol, but some could be extracted with hot ethanol.

In most instances, after elution and extraction, some extractives could not be accounted for. This varied from the extremes of 5.1% for Layer 4 to 19.0% for Layer 1 with an average loss of 11.5% observed. Sorption of extractives on the polyamide was the most probable explanation for this loss. No adverse effects on the fractionating power of the polyamide were observed by sorption of the extractives.

COMPARISON WITH PREVIOUS WORK

Most of the glucosides and other components isolated, through elution polyamide column chromatography, and identified in this study were found in previous work on the lead subacetate analysis of P. grandidentata bark hot water extractives (5, 7). Salicin was found in relatively high yields (0.27%). However, following lead subacetate treatment during the present study, it was obtained in much greater quantities (0.61%) probably because of the fractionation on a polyamide column. In past work on extractives from various species not treated with lead subacetate, most yields of salicin were not as high as that

TABLE XII

EXTRACTIVE MATERIAL BALANCE

	Layer 1	Layer 2	Layer 3	Layer 4	Condensate
Weight of extractives placed on the columns, g.	1.31	30.3	36.4	17.6	2.93
Weight of extractives fractionated					
Elution with water, g.	0.44	19.7	23.5	12.1	2.06
Gradient elution, g.	–	8.6	9.6	–	–
Weight of extractives[a] removed by extraction, g.	0.62	2.2	0.8	4.6	0.52
Total, g.	1.06	30.5	33.9	16.7	2.58
Difference, %	-19.0	+0.65	-6.87	-5.1	-11.9

[a]Extractives removed from the polyamide by hot water and/or hot ethanol.

TABLE XII (Continued)

EXTRACTIVE MATERIAL BALANCE

	Whole Bark-1	Whole Bark-2	Whole Bark after Treatment with Lead Subacetate
Weight of extractives placed on the columns, g.	35.4	35.4	16.2
Weight of extractives fractionated			
Elution with water, g.	21.1	21.9	12.7
Gradient elution, g.	-	8.3	-
Weight of extractives[a] removed by extraction, g.	7.9	0.9	1.2
Total, g.	29.0	31.1	13.9
Difference, %	-18.2	-12.2	-14.2

[a]Extractives removed from the polyamide by hot water and/or hot ethanol.

obtained in this study indicating that P. grandidentata bark is a relatively good source of salicin (14, 15, 21).

Pearl and Darling (15) identified 1-O-p-coumaroyl-β-D-glucose while working with triploid P. tremuloides bark. They reported a yield of "crystalline" material (0.18%) which agrees closely with the yield found during the present investigation (0.20%). A small amount of this component was found in earlier work on P. grandidentata by Pearl, et al. (5) but remained unidentified until their work on the triploid species.

Grandidentatin always crystallized from solution in conjunction with grandidentoside during the present study. Because previous work on P. grandidentata (6) involved treatment with lead subacetate, grandidentoside was not observed. In this work grandidentatin was available in yields of 0.07% merely by working with the crude extracts. Polyamide column chromatography of the clarified extracts in the present study showed grandidentatin to be present to a lesser degree (0.02%). However, only trace amounts were identified in a September bark of P. grandidentata (7) indicating that grandidentatin is not always present in substantial yields. Since grandidentatin and grandidentoside were usually found together, determination of amounts recovered in each case was difficult. An estimate from the whole bark-2 elution data indicates that the yield of grandidentatin was about 0.06% and that of grandidentoside was about 0.09%.

Tremuloidin was found in earlier lead subacetate-clarified P. grandidentata bark extractives (5). The present investigation showed that, for the liberation of all tremuloidin, such a treatment was necessary.

The salireposide yield was comparable with or slightly lower than the amounts reported for other species (13-15, 18, 21).

Pearl and Darling (21) reported pyrocatechol and salicyl alcohol yields of 0.08% in P. balsamifera bark. Using the same methods of identification for P. grandidentata bark in the present study, the pyrocatechol yield was 0.10% while that for salicyl alcohol was only 0.005%.

IDENTIFICATION OF POPULOSIDE

A new glucoside was isolated by gradient elution polyamide column chromatography. This new component, melting at 186-188°C., has a slight yellow appearance. On a silica gel thin-layer chromatogram developed in 4:1 chloroform-methanol (trace of acetic acid), sprayed with sulfuric acid, and heated, it exhibited a lavender streak having an R_f of 0.32. Use of a stronger acid, formic rather than acetic in the developing solvent, eliminated the streaking and produced a single lavender spot at the same R_f. Free phenolic hydroxyls were suggested by a color reaction with diazotized p-nitroaniline. This new compound has been designated "populoside," and its structure has been established as the ω-caffeic acid ester of salicin [o-(3,4-dihydroxycinnamoyloxy-methyl)phenyl β-D-glucopyranoside].

Populoside was acetylated with acetic anhydride and pyridine, and the resulting acetate was subjected to analysis and mass spectrometry. This tool has played a major role in the identification of this component (16-18, 43). The mass to charge ratio (m/e) of all important peaks for this compound are given in Table XXV (Appendix III).

The mass spectrum (Fig. 14) shows a molecular ion of 700, corresponding with populoside hexaacetate (XI). A prominent peak at m/e 331 suggested that

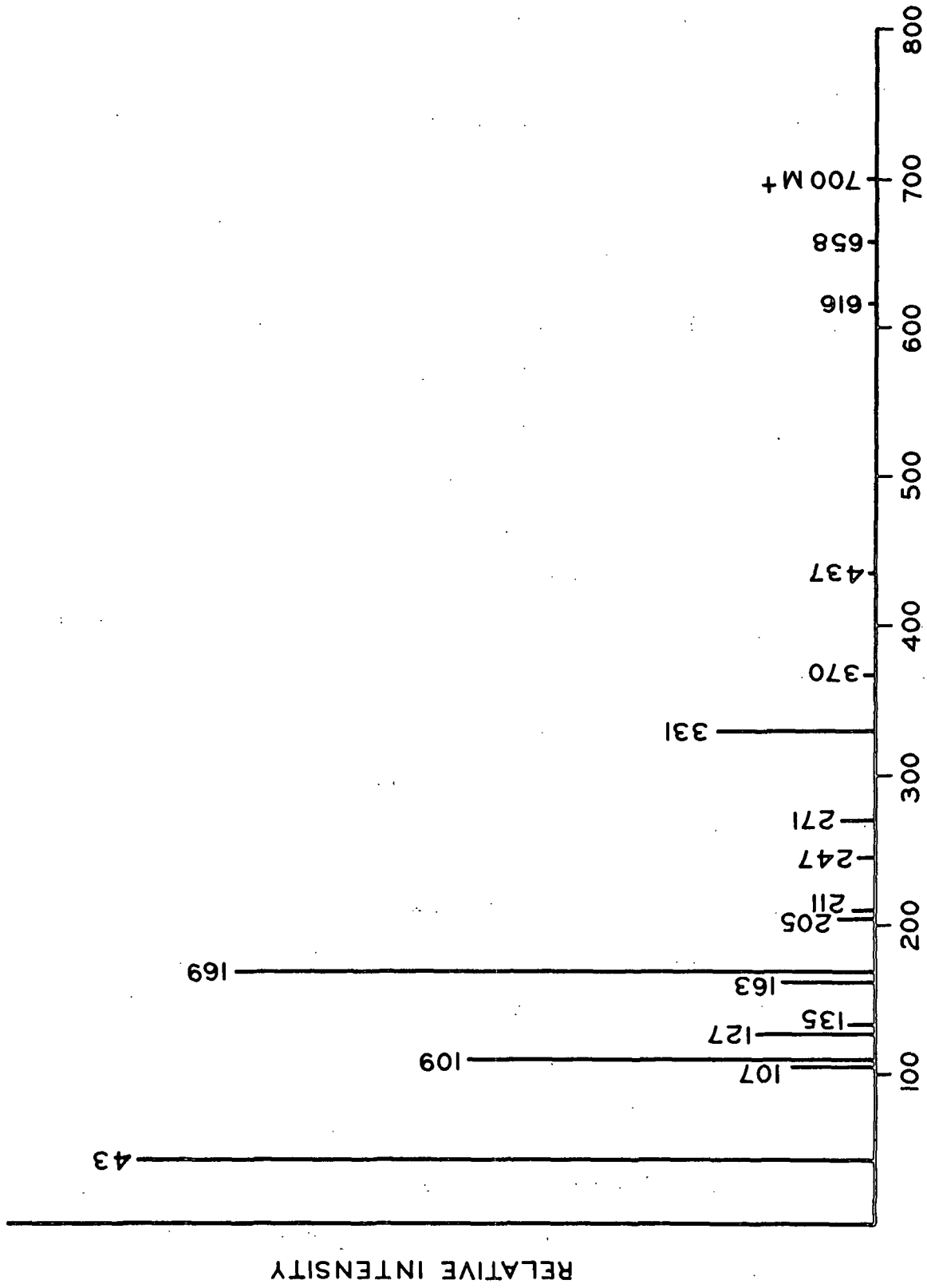


Figure 14. Mass Spectrum of Populoside Hexaacetate

this compound undergoes primary fragmentation at the glucosidic linkage, resulting in the formation of a tetraacetylglucose oxonium ion (XII) (Fig. 15a). This latter ion was originally reported by Bieman and coworkers (44) for the primary fragmentation of glucose pentaacetate. Pearl and Darling (16) also found it to be present in the fragmentation patterns of the acetates of all glucosides without substitution on the glucose moiety. Other related glucose ions (m/e 271, 211, 169, and 109) were present in abundance. Structures corresponding with these peaks proposed by Smale and Waight (45) and Heyns and Mueller (46) are also illustrated in Fig. 15a. The latter individuals, however, did not show the degradation of the m/e 169 ion to m/e 127 by loss of ketene. This sequence is accounted for by a strong m/e 127 peak in the spectrum.

Previous work (16) indicated that acetylated phenolic hydroxyls are unstable and ketene (mol. wt. 42) often was split before fragmentation of the C-1 linkage occurred. Since peaks were located at m/e 658 and m/e 616, loss of one and two ketene groups, respectively, a dihydroxy phenolic moiety was indicated (Fig. 15b).

Another fundamental ion pertinent in the identification of populoside corresponded with the acetylated caffeoyl ion (XIII) at m/e 247 (Fig. 15b). Associated with this ion were peaks at m/e 205 and m/e 163 representing the loss of ketene from the phenolic hydroxyls. The m/e 135 peak corresponded with the elimination of carbon monoxide from the deacetylated caffeoyl ion.

The presence of peaks at m/e 437 and m/e 370 suggested the ions indicated in Fig. 15c, further substantiating the proposed structure of populoside.

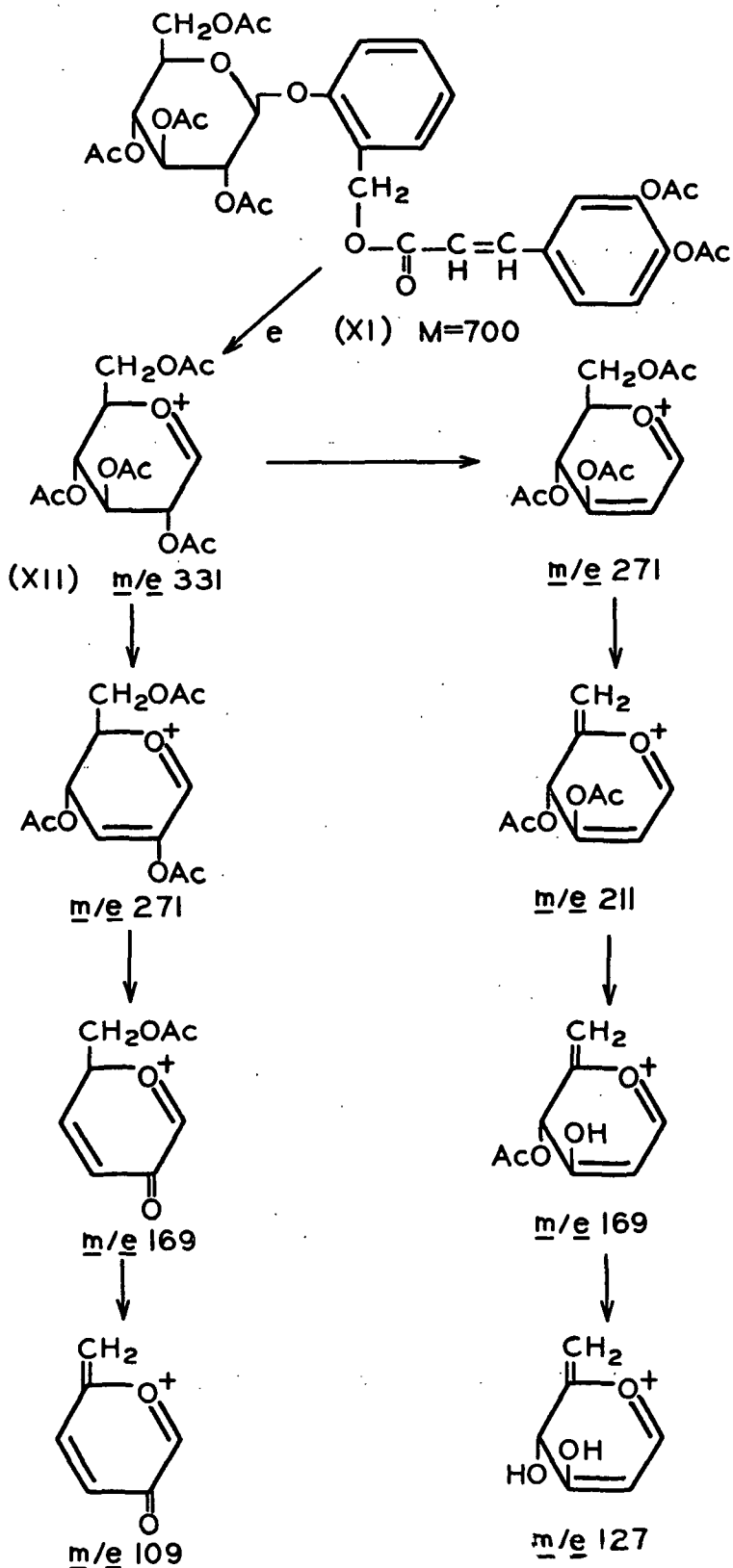


Figure 15a. Mass Fragmentation Pattern of Populoside Hexaacetate

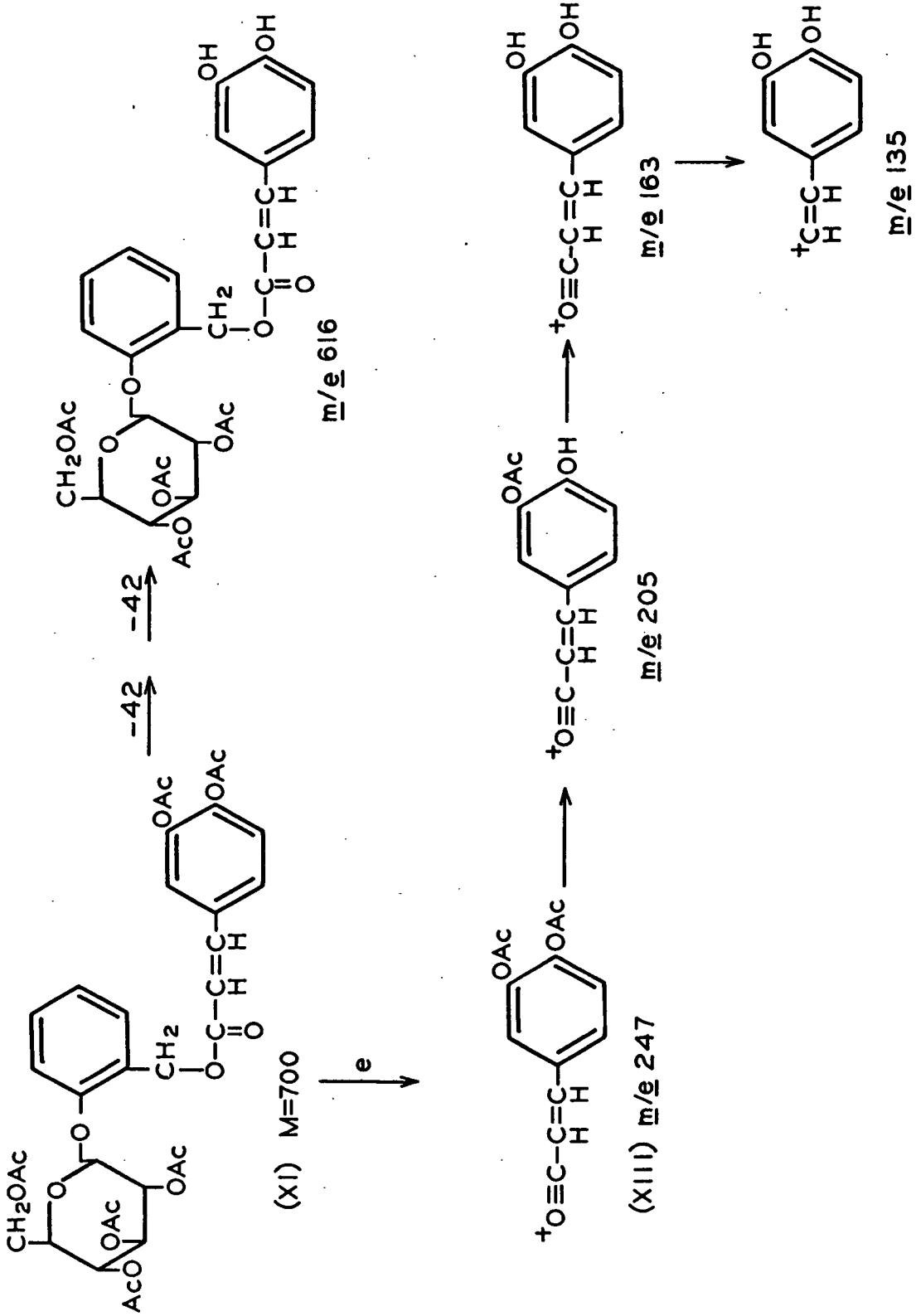


Figure 15b. Mass Fragmentation Pattern of Populoside Hexaacetate

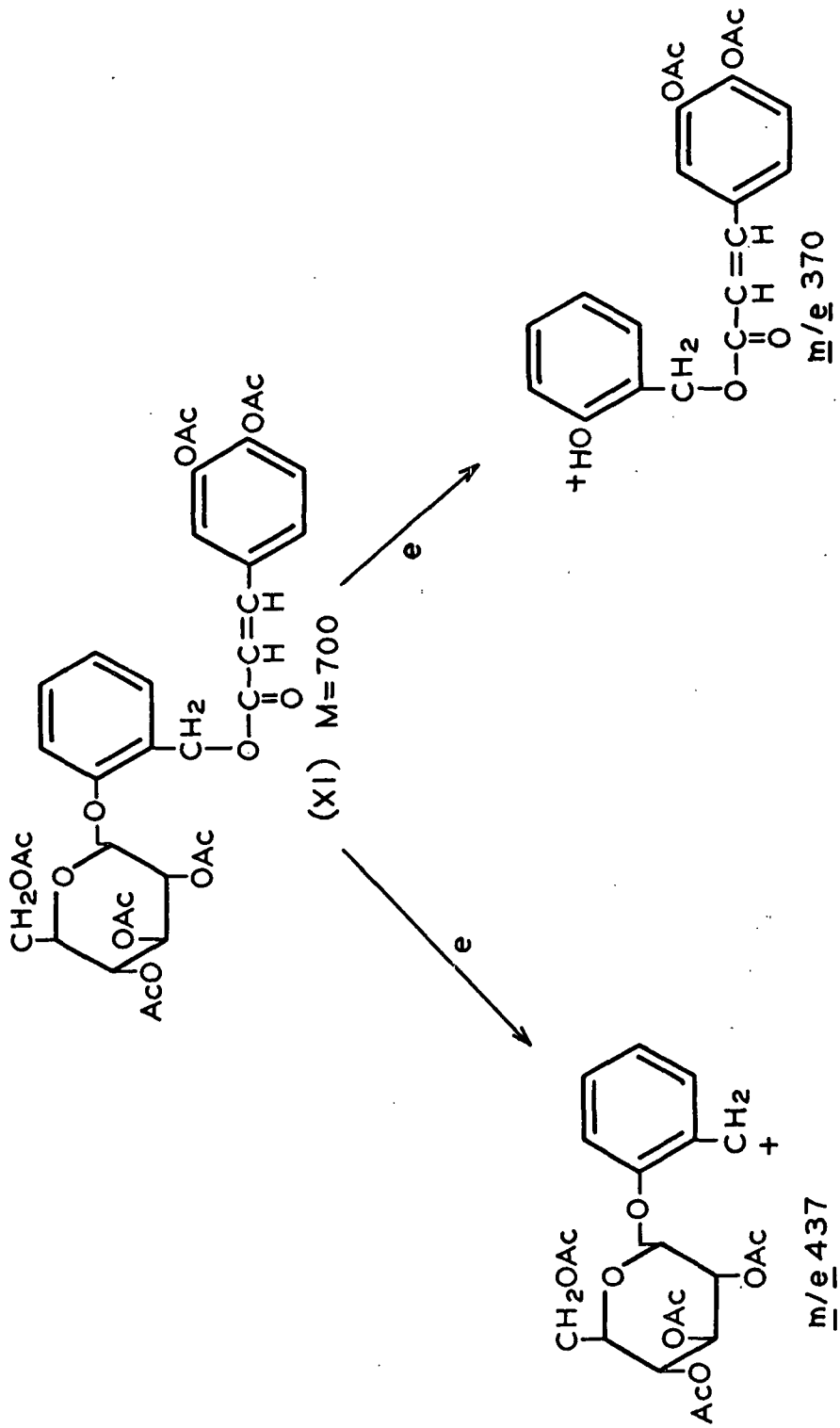


Figure 15c. Mass Fragmentation Pattern of Populoside Hexaacetate

Further proof of structure was accomplished by enzymatic and alkaline hydrolysis of the parent molecule. Hydrolysis of populoside with beta-glucosidase yielded glucose which was identified by chromatographic techniques.

Saponification with alkali yielded crystalline salicin and caffeic acid. The salicin, melting at 198-199°C., did not depress a mixed melting point with authentic salicin; both compounds exhibited similar characteristics with sulfuric acid on a silica gel chromatoplate.

The organic acid melting at 213-215°C. with decomposition had an infrared absorption spectrum (Fig. 41-Appendix VI), gas chromatographic retention times, and characteristics on paper chromatograms identical with that of known caffeic acid. The melting point of 213-215°C. corresponded with previous (47) melting points of caffeic acid. Positive supplementary tests characteristic of authentic caffeic acid (48) were also exhibited by the organic acid.

IDENTIFICATION OF GRANDIDENTOSIDE

Another new glucoside was isolated in admixture with grandidentatin between Fractions 60 and 80 when ethyl acetate extractive samples were subjected to elution chromatography with water on a polyamide column. The mixture on a silica gel thin-layer chromatogram with sulfuric acid exhibited two spots: a slate-green spot at an R_f of 0.43 indicating grandidentatin, and a lavender streak having an R_f of 0.35. Again use of formic acid in the developing solvent eliminated streaking. This new compound has been designated "grandidentoside," and its structure has been shown to be a caffeic acid ester of cis-2-hydroxy-cyclohexyl β-D-glucopyranoside. Mass spectrometry and nuclear magnetic resonance spectrometry strongly suggested substitution at the C-2 position on the glucose moiety.

Separation of grandidentatin and grandidentoside was accomplished on a silica gel column using a chloroform-methanol-formic acid solvent. The methanol concentration was initially quite low but gradually was increased to induce a gradient within the column. Complete separation of the two components was achieved with little or no degradation.

From a water solution of grandidentoside containing a small amount of methanol and formic acid, a small yield of crystalline grandidentoside melting at 196-197°C. was obtained. If a solution of grandidentoside was not kept slightly acidic, degradation was soon observed. Acetylation was accomplished by the addition of acetic anhydride and pyridine to a sample of grandidentoside sirup. The resulting acetate was subjected to analysis by mass spectrometry. Mass to charge ratios (m/e) of all important peaks for the spectrum of this acetate are given in Table XXVI (Appendix III).

The mass spectrum (Fig. 16) showed a molecular ion of 692, corresponding with the proposed structure of grandidentoside hexaacetate (XIV). A dihydroxy phenolic moiety again was evident by peaks at m/e 650 and m/e 608 representing the loss on one and two ketene groups, respectively. From the relative abundance of ions, the primary fragmentation route (Fig. 17a) appeared to be through the splitting of the caffeoyl group (XIII) (m/e 247) with the subsequent loss of two ketene groups (m/e 205 and m/e 163). It is interesting to observe that the deacetylated caffeoyl ion (m/e 163) readily lost a proton to form an ion-free radical combination (XV) (m/e 162). The former has a relative intensity of 89.4% while the latter combination has a somewhat lower relative intensity (63.7%). This same phenomenon was noted for grandidentatin (40).

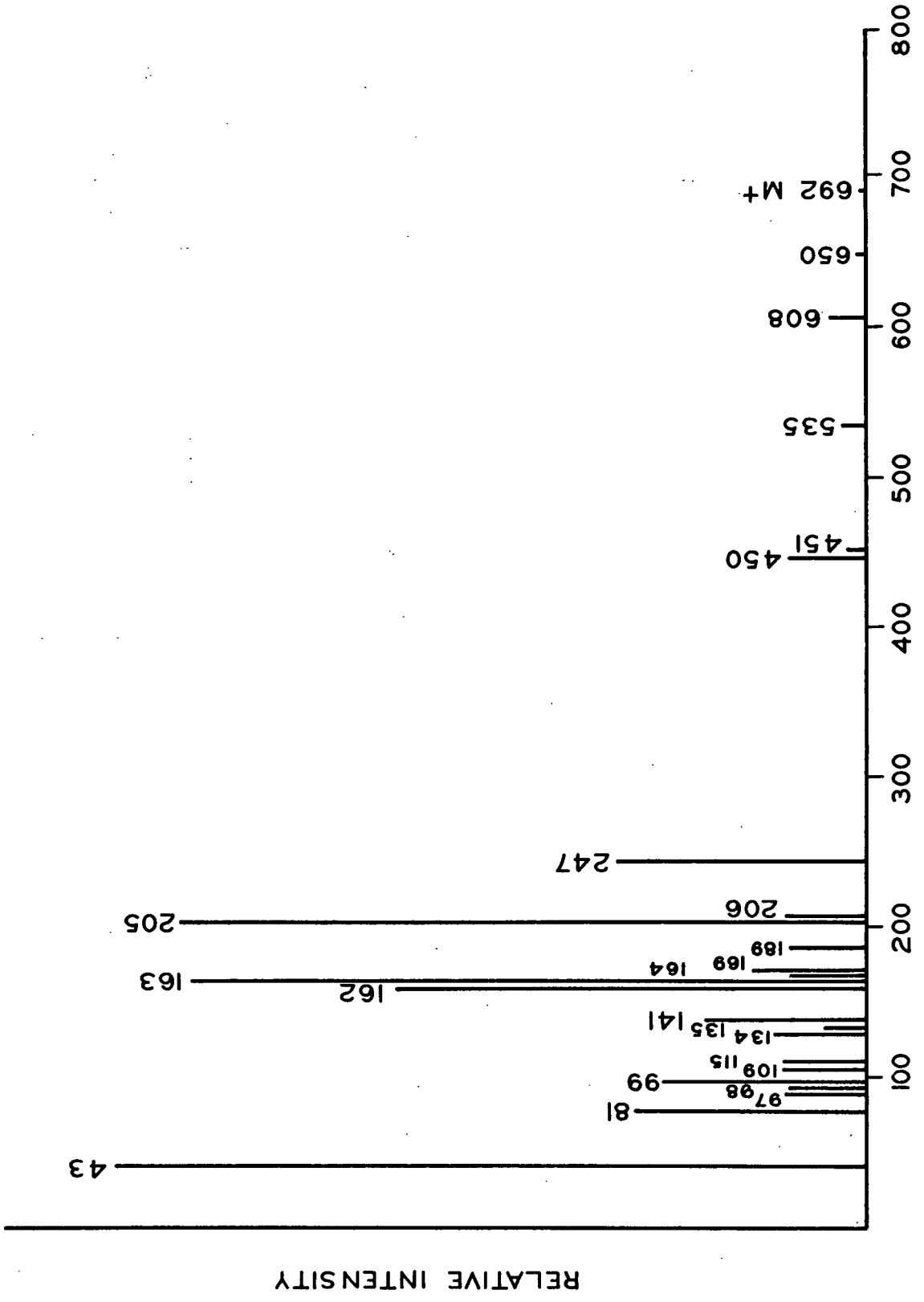


Figure 16. Mass Spectrum of Grandidentoside Hexaacetate

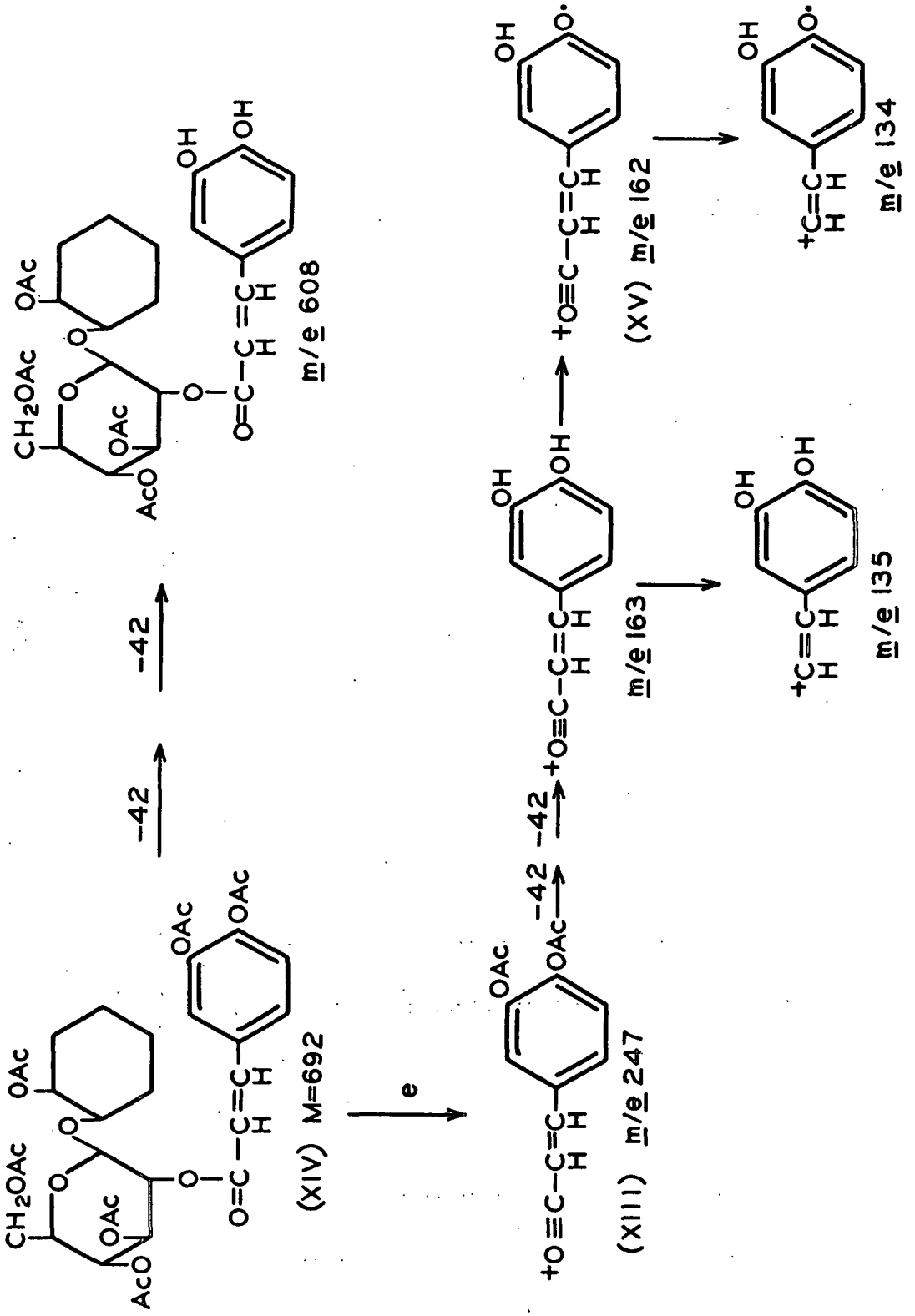


Figure 17a. Mass Fragmentation Pattern of Grandidentoside Hexaacetate

Another important peak which corresponded with the caffeic acid ester of the triacetylglucose oxonium ion (XVI) was at m/e 535. This and structures resulting from the loss of ketene are depicted in Fig. 17b. Absence of a dominant peak at m/e 331 showed that the caffeoyl group was attached to the glucose moiety and not to the cyclohexyl group.

Further proof of structure was accomplished by alkaline hydrolysis of grandidentoside. Saponification yielded a chromatographically pure compound which appeared to be authentic grandidentin (cis-2-hydroxycyclohexyl β-D-glucopyranoside). Caffeic acid was also evident by thin-layer chromatography. Acetylation of the purified hydrolyzate with acetic anhydride and pyridine yielded crystals which melted at 120-121°C., did not depress a mixed melting point of, and had an infrared absorption spectrum (Fig. 40-Appendix VI) identical with that of known grandidentin acetate.

The combination of mass spectrometry and alkaline hydrolysis demonstrated that grandidentoside was composed of caffeic acid and grandidentin, and that substitution was on the glucose moiety. This solved the structure of grandidentoside except for location of the caffeoyl group on glucose. With only a small amount of material, location of the caffeoyl group by methylation was impractical. A periodate oxidation study would not be conclusive. Consequently, attention was focused on nuclear magnetic resonance (NMR) spectrometry. Strong evidence for substitution of the caffeoyl group at the C-2 position of glucose was suggested by NMR. Details for the application of this technique in the analysis of phenolic glucosides is developed below.

APPLICATION OF NUCLEAR MAGNETIC RESONANCE SPECTROMETRY IN THE ANALYSIS OF PHENOLIC GLUCOSIDES

Nuclear magnetic resonance spectrometry has proven to be useful in the field of carbohydrate research and has been discussed in a general review (49).

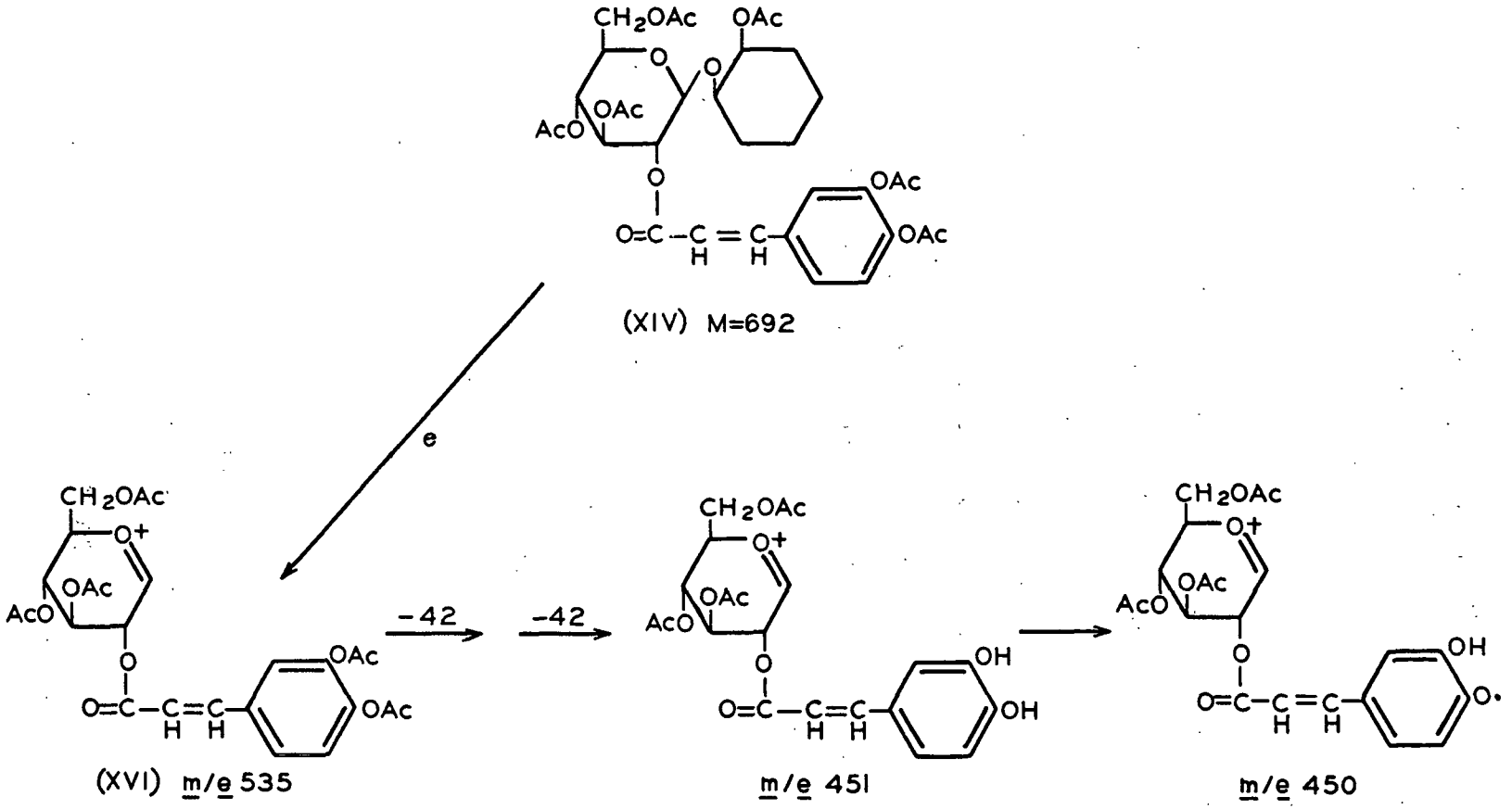


Figure 17b. Mass Fragmentation Pattern of Grandidentoside Hexaacetate

NMR has been used extensively for determining the configuration of unknown carbohydrates in solution. However, very little has been done with respect to the specific substitutional pattern of groups on the carbohydrate moiety, and almost no work has been reported on phenolic glucosides similar to those being studied in this thesis.

Before attempting to locate the caffeoyl group in grandidentoside by NMR, several phenolic glucosides with established structures were examined. These included salicin, populin, tremuloidin, salicyloyl salicin, grandidentin, and grandidentatin. All of the NMR spectra were determined in a deuterated methyl sulfoxide solution. Exchange of the hydroxyl protons was accomplished with deuterium oxide. Chemical shifts were reported in parts per million [p.p.m.] (τ). Only that portion of the NMR spectrum pertinent for an understanding and determination of the structure of grandidentoside has been presented in this discussion. The region of primary interest extends from a chemical shift of 4.0 to 6.0 p.p.m. (τ).

In the NMR spectrum of salicin (Fig. 28-Appendix IV), three protons were observed between 5.0 and 5.7 p.p.m. The anomeric proton (H-1) of glucose appeared as a masked doublet at 5.10 p.p.m. with a coupling constant of 7 c.p.s. For the remaining two protons, corresponding with the omega methylene protons of the benzyl group, an AB quartet was observed. The two protons were located at 5.32 and 5.42 p.p.m. with a coupling constant of 15 c.p.s. Nonequivalent methylene protons were not expected since both benzyl alcohol (50) and salicyl alcohol exhibited a strong singlet for these protons at 5.45 p.p.m. Hindered rotation as a result of a "locked conformation" is the most probable explanation for this phenomenon. A locked conformation could result from intramolecular hydrogen bonding (Fig. 18). This type of hydrogen bond has been suggested

before (51). In methyl sulfoxide, intramolecular hydrogen bonding can exist even though most of the glucose hydroxyls are strongly hydrogen bonded to the solvent (52).

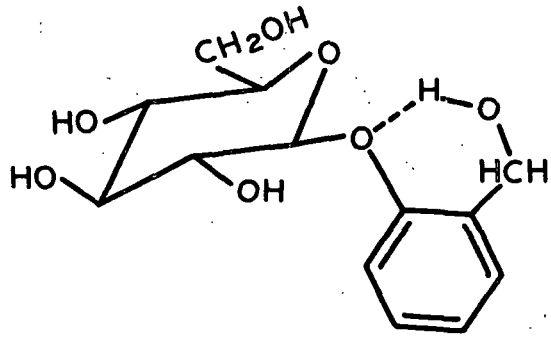


Figure 18. Postulated Hydrogen Bonding in Salicin

Substitution at the omega position, as in salicyloyl salicin (Fig. 29-Appendix IV), eliminated the AB pattern producing a singlet at 4.45 p.p.m., which corresponded closely with the singlet observed in benzoyl benzoate (50). Collapsing of the quartet to a singlet by substitution provided additional evidence that the benzylic hydroxyl proton could be involved in intramolecular hydrogen bonding.

Tremuloidin, 2-O-benzoylsalicin, exhibited four protons in the region of fundamental interest (Fig. 30). Again an AB quartet was observed for the omega methylene protons. However, the difference in chemical shifts increased from 0.10 to 0.24 p.p.m. (protons at 5.55 and 5.79 p.p.m.) while the coupling constant remained at 15 c.p.s. These data indicated that substitution at C-2 forced the omega methylene protons into environments even different than

in salicin. The remaining two protons were attributed to the anomeric proton (H-1) and the H-2 proton. Esterification of a ring hydroxyl causes the corresponding ring proton to shift downfield by 1.0 to 1.2 p.p.m. (49). Benzoylation at C-2 shifted H-2 into the region of 4.8 p.p.m. Substitution at C-2 also shifted the anomeric proton slightly downfield. Similar results have been observed for ethyl β-D-glucopyranosides (53) as indicated in Table XIII.

TABLE XIII

PARTIAL NMR SPECTRA OF ETHYL β-D-GLUCOPYRANOSIDES[a]

Proton	Chemical Shift, p.p.m.
Ethyl 3,4,6-tri-O-methyl β-D-glucopyranoside	
H-1	5.78
H-2	–
Ethyl 2-O-acetyl 3,4,6-tri-O-methyl β-D-glucopyranoside	
H-1	5.66
H-2	5.2

[a]Determined in CCl_3D.

The NMR spectrum of populin, 6-O-benzoylsalicin, agreed with the spectra previously discussed (Fig. 31). The anomeric proton again was observed at about 5.1 p.p.m. and an AB quartet similar to that for salicin was visible. Since integration yielded exactly five protons between 4.0 and 6.0 p.p.m., the remaining two protons could be attributed to the C-6 methylene protons brought downfield by esterification.

Nuclear magnetic resonance spectrometry data for salicin and salicin derivatives led to the conclusion that substitution at C-2 or C-6 could be distinguished by the number and nature of the protons observed in the region of 4.0 to 6.0 p.p.m. Substitution at C-2 only shifted one proton downfield by esterification whereas substitution at C-6 shifted two protons downfield. Although no work was done on derivatives substituted at C-3 or C-4, other work on acetylated methyl β-D-glucopyranosides indicated that if either of these positions were esterified, the corresponding ring protons would be shifted downfield slightly more than those of C-2 or C-6 (54). Since glucosides of natural products are substituted usually in the latter two positions, NMR appears to be valuable in distinguishing the nature of substitution on a glucose moiety. Mass spectrometry indicates whether or not the glucose is substituted.

The common component of both grandidentatin and grandidentoside, grandidentin, has a partial NMR spectrum depicted in Fig. 32. In this class of compounds, the region of interest was narrower and extended from 5.0 to 6.0 p.p.m. A doublet corresponding with the anomeric proton of grandidentin appeared at 5.67 p.p.m., with a coupling constant of 7 c.p.s., compared with 5.91 and 5.72 p.p.m. for methyl β-D-glucopyranoside and cyclohexyl β-D-glucopyranoside, respectively.

Grandidentatin (Fig. 33) showed two protons between 5.0 and 6.0 p.p.m. Esterification at C-2 shifted the H-2 proton downfield by 1.0 to 1.2 p.p.m. and also shifted the anomeric proton downfield slightly. Of specific interest was the spike located at 5.36 p.p.m. and the general shape of the peaks.

Since there is only a slight difference between the p-coumaroyl and caffeoyl moieties, if substitutions were in identical positions, similar NMR

spectra would be anticipated. Grandidentoside has a partial NMR spectrum shown in Fig. 34. Although the background noise was greater due to a smaller sample size, integration yielded exactly two protons; a strong spike was observed at 5.36 p.p.m. The basic shape of the peaks also corresponded with those observed for grandidentatin. Even more illustrative is Fig. 19 which compares the NMR spectrum of grandidentatin with the spectrum of an equal mixture of the two components. The aromatic protons of grandidentatin are visible in both spectra between 2.0 and 4.0 p.p.m.; however, the lower spectrum is complicated by the presence of grandidentoside. It is evident that the same peaks and splitting patterns are present in both spectra for the protons of interest between 5.0 and 6.0 p.p.m., indicating identical substitutional patterns for the two compounds.

From these data it was concluded that grandidentoside, like grandidentatin, is substituted at the C-2 position on the glucose moiety.

CAFFEIC ACID DERIVATIVES

Isolation and identification of populoside and grandidentoside, complex glucose esters of caffeic acid, has added to the list of known caffeic acid glucosides. Cinnamic acids occur in combined form in nearly every higher plant (55), primarily as esters (56). Although caffeic acid is the most widely reported (57), only a few caffeic acid glycosides are known (Table XIV) (55). This fact probably was a result of the fractionation methods previously employed. Treatment with lead subacetate, for example, eliminated grandidentoside and possibly other caffeic acid derivatives from the extractive mixture. The techniques employed in the present study allowed for the isolation of these components and may be beneficial in future work where caffeic acid derivatives are of interest.

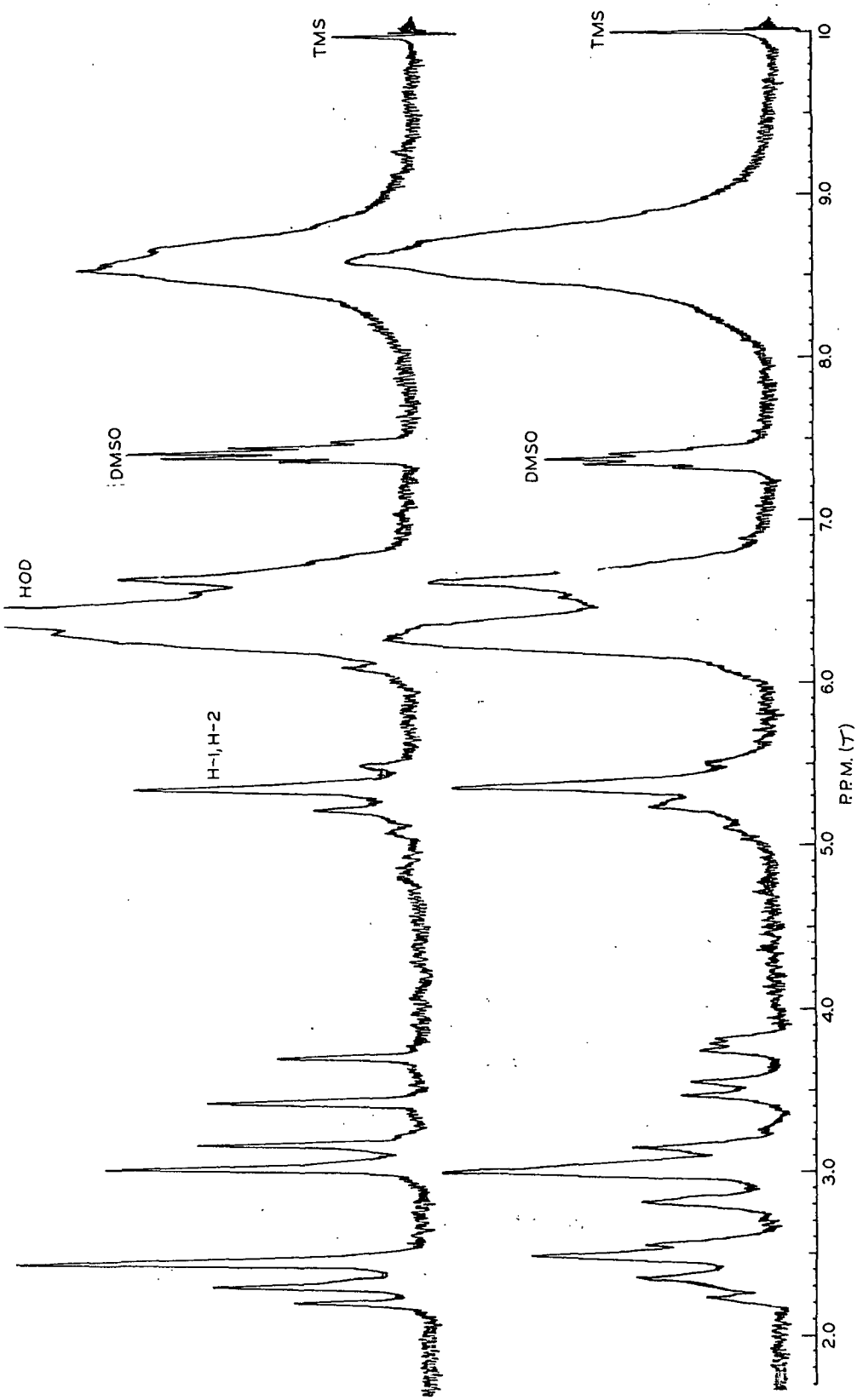


Figure 19. NMR Spectra of Grandidentatin (Top) and of an Equal Mixture of Grandidentatin and Grandidentoside (Bottom) (60 MHz) in Methyl Sulfoxide-d_6 Containing Deuterium Oxide. The Mixture also Contained a Trace of Hydrochloric Acid

TABLE XIV

KNOWN CAFFEIC ACID GLYCOSIDES IN PLANT TISSUE[a]

1-O-Caffeoyl glucose (XVII)

1-O-Caffeoyl gentiobiose (XVIII)

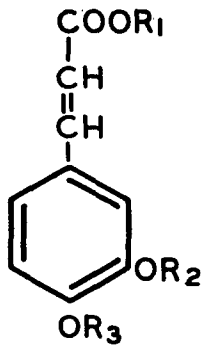
Caffeic acid 3-β-D-glucoside (XIX)

Caffeic acid 4-β-D-glucoside (XX)

Echinacoside[b] (XXI)

[a]Refer to Fig. 20.

[b]Refer to Fig. 21.



(XVII) R_1 = β-D-GLUCOSE; R_2=R_3=H

(XVIII) R_1 = 6-O-β-D-GLUCOPYRANOSYL-β-D-GLUCOSE
R_2=R_3=H

(XIX) R_1=H; R_2 = β-D-GLUCOSE; R_3=H

(XX) R_1=H; R_2=H; R_3 = β-D-GLUCOSE

Figure 20. Caffeic Acid Glucosides

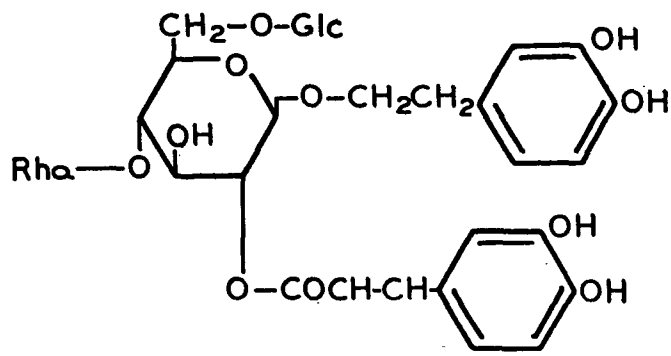


Figure 21. Structure of Echinacoside

Orobanchin, a complex caffeic acid derivative containing rhamnose and glucose among other constituents, is known to exist, but its structure has not been determined (55). Other caffeic acid combinations are reported, e.g., chlorogenic and chicoric acids; however, these are not glycosides.

In a review, Neish (58) indicated that there are good reasons to believe that cinnamic acids, such as caffeic, play a central role in the biosynthesis of lignin. Goldschmid and Hergert (59) found, as the present author has, that glycosides of ligninlike compounds are not restricted to a specific region of the bark. In accordance with Goldschmid and Hergert, these compounds possibly are intermediates in the formation of extractive components and are not involved in lignin synthesis. Alternatively, they are extraneous and possess no important biological function in normal tissue (60).

SILICA GEL COLUMN CHROMATOGRAPHY

Fractions from the polyamide column chromatography of the ethyl acetate extractives still were quite complex (Fig. 3). After a major separation on polyamide and a combination of fractions containing similar components, alternate chromatographic procedures were necessary to accomplish any subsequent separation. Since grandidentatin and grandidentoside were separated successfully by silica gel column chromatography, an attempt was made to separate one of these complex fractions. Extractives from Fractions 61 to 85 (Fig. 3), containing eight major components, were eluted on a silica gel column with a chloroform-methanol-formic acid solvent. The concentration of methanol was increased gradually to induce a gradient within the column.

As observed in Fig. 22, a separation of each component was achieved. These data indicated that by using this technique, the extractives after polyamide chromatography can be fractionated further into individual components.

When employed on a large scale, it should be possible to isolate most every component indicated by thin-layer chromatography. In conjunction with mass spectrometry and other analytical tools, future investigators should be able to determine the identity of many new compounds.

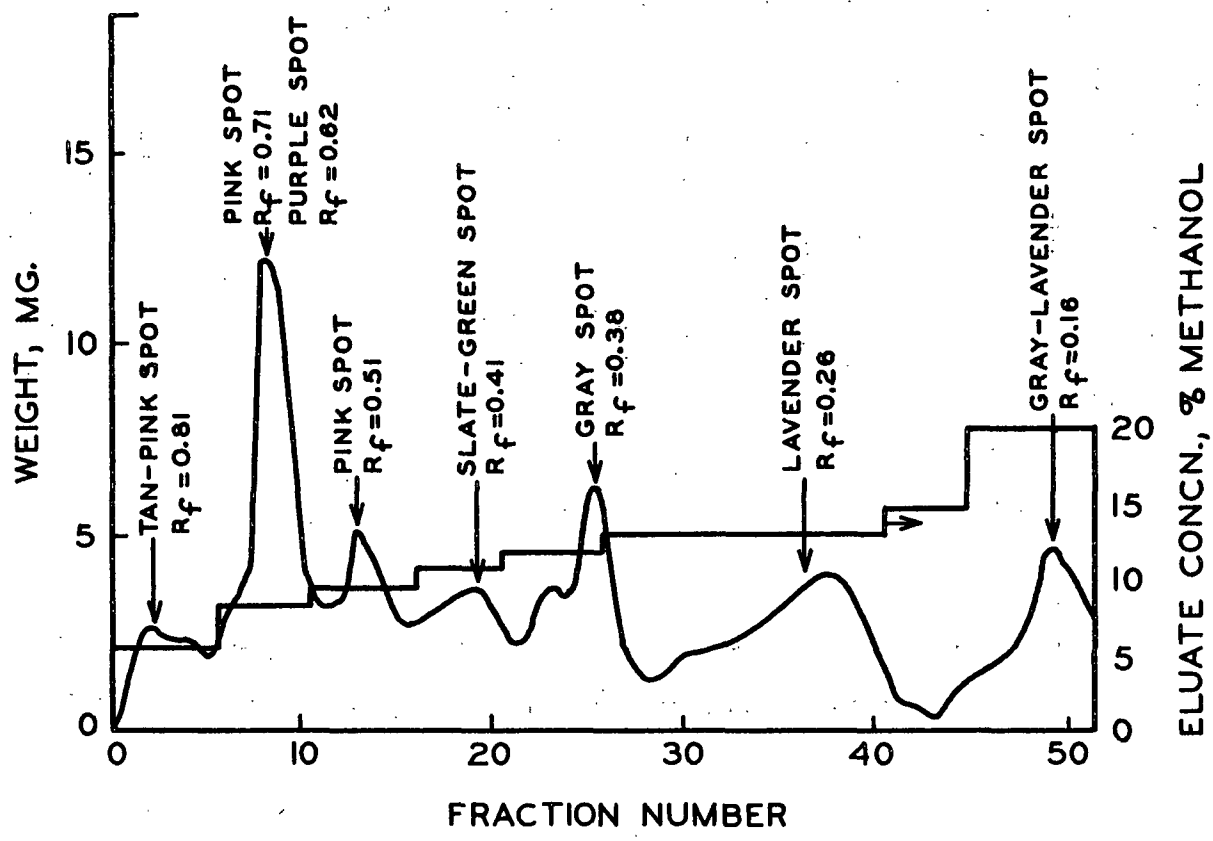


Figure 22. Silica Gel Column Chromatography of the Whole Bark Ethyl Acetate Extractives (Fractions 61-85) After Polyamide Column Chromatography

EXPERIMENTAL PROCEDURES

STARTING MATERIAL

Three Populus grandidentata, bigtooth aspen, trees with smooth green bark were cut on May 11, 1967, ten miles northwest of Green Bay, Wisconsin. All trees appeared to be from the same clone and were from 14 to 15 years of age.

SEPARATION OF THE BARK INTO ITS ANATOMICAL LAYERS

One to two foot bolts of the rather smooth upper portion (3-4 inches in diameter) of each tree were steamed in a digester at 20 p.s.i. for 15 minutes and then debarked by a procedure described by Hossfeld and Hunter (3).

LAYER 1

The outermost layer, phellem, was peeled and scraped away with a dull knife. Before steaming, this layer was a pale gray-green; during steaming it became a dark brown.

LAYER 2

Following removal of the phellem, the second or green pulpy layer, phelloderm, was scraped down to a dense crystallinelike region of the secondary phloem.

LAYER 3

To separate the secondary phloem, inner bark, into two layers, the bark was cut into two-inch strips along the length of each bolt. As each strip was removed, the innermost fibrous layer was peeled away until the dense outer region of the secondary phloem was reached. This outer region was designated as Layer 3.

LAYER 4

The innermost fibrous layer which was removed from the secondary phloem as indicated above was labelled Layer 4.

Since steaming may have removed hot water soluble components, the "condensate" from the digester during the steaming of several bolts was collected, concentrated, and analyzed in conjunction with the tissue layers.

In addition to the whole unsteamed bark, each tissue layer was air dried, Wiley milled, and stored in polyethylene bags for future use.

A cross-section photomicrograph of the bark used in this study (Fig. 1) was obtained by mounting a sample in Canada balsam, cutting a 15-μm. section, and staining with safranin and iron alum hematoxylin*.

DISTRIBUTION OF EXTRACTIVES IN POPULUS GRANDIDENTATA BARK

Duplicate samples of each tissue layer and of the whole bark were extracted continuously and successively in a Soxhlet extractor for a minimum of eight hours with the following solvents: low-boiling petroleum ether (30-60°C.), ethyl ether, benzene, absolute ethanol, and hot water. The hot-water extraction was conducted in the continuous extractor illustrated in Fig. 23.

About 10 g. of ovendry solids were used for analysis in each case, except for Layer 1, where about 2 g. were used.

*Work performed by Mr. J. O. Hankey of The Institute of Paper Chemistry.

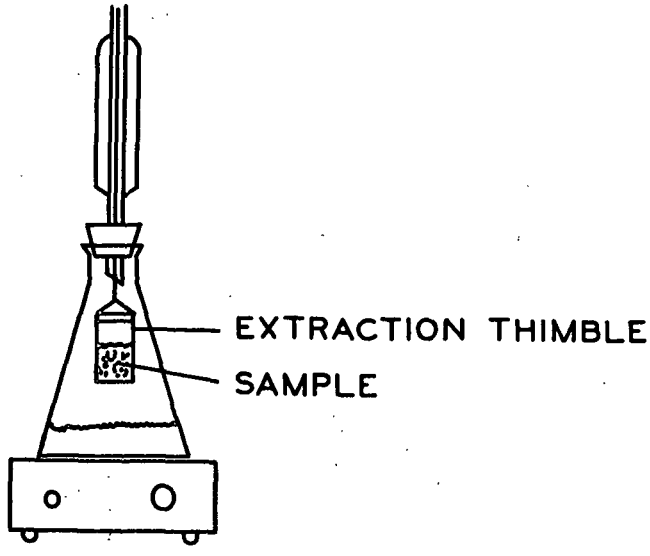


Figure 23. Continuous Hot Water Extraction Device

SOLUBLE SUGAR ANALYSIS*

Identification of sugars was based on the R_G values when paper chromatograms were developed in 8:2:1 ethyl acetate-pyridine-water (EPW) on Whatman No. 1 paper. The sugars were detected through the use of a modified silver-spray procedure (61). Secondary hydrolysis of the hot water extract was accomplished as described by Saeman, et al. (62).

Sucrose and maltose were alternatively identified as the trimethylsilyl derivatives by gas chromatography in conjunction with the specific conditions listed in Table XV.

*Determined by the Analytical Department of The Institute of Paper Chemistry.

TABLE XV

CONDITIONS EMPLOYED FOR THE IDENTIFICATION OF SUCROSE AND MALTOSE AS THE TMS DERIVATIVES BY GAS CHROMATOGRAPHY

Gas chromatograph	Aerograph Model 1520
Column	3% SE 52 on Chrom W (80/100) SS
Column length	6 ft.
Column diameter	1/4 in.
Column temperature	255°C.
Gas flow rate	40 cc./min.
Retention times	
Sucrose	16.2 min.
Alpha-maltose	18.3 min.
Beta-maltose	20.2 min.

ANALYSIS OF THE EXTRACTIVE FREE BARK*

In addition to the whole bark, extractive-free samples from each of the tissue layers were subjected to analysis for concentrations of alpha-cellulose, holocellulose, ash, material insoluble in 72% sulfuric acid, and methoxyl content. The methods used for these analyses are summarized below.

1. Removal of lignin prior to alpha-cellulose determination — Institute Method 28.
2. Alpha-cellulose — Institute Method 421.
3. Holocellulose — Institute Method 28.
4. Insoluble material in 72% sulfuric acid — TAPPI T 13 m-54.
5. Methoxyl content — Institute Method 18.

ANALYSIS OF THE HOT WATER EXTRACTIVES

Duplicate samples of the tissue layers and a single sample of the whole unsteamed bark were extracted with hot water using a method described by

*Determined by the Analytical Department of The Institute of Paper Chemistry.

Pearl, et al. (5). The ovendry weight of each layer extracted was based on a whole-bark sample of 1500 g. and on the relative contribution of each layer. All fractions were suspended in a volume of deionized water, brought to boiling, and kept at boiling with continuous agitation for a period of time. Table XVI lists the volume and time used for each sample. In each case the hot slurry was filtered with suction through a cloth, and the residue was resuspended in an equivalent volume of water. For the first set of extractions of the tissue layers, this process was repeated three times. To remove any undissolved material, the filtrates, which were kept separate, were filtered (warm) through a Celite* pad. Every fraction was concentrated in a circulating evaporator operating under reduced pressure. A solids analysis was made on each of the three extractions for the four fractions to determine how much material was removed (Table XV).

The second series of extractions of the four layers and of the whole bark involved four repetitions of the extraction process. A summary of these data is also found in Table XV. In the latter case the filtrates were combined, filtered, and concentrated so only a total extractive weight was obtained.

EXTRACTION WITH ETHYL ACETATE

The concentrated water extracts were extracted with ethyl acetate in a nonagitated liquid-liquid extraction apparatus, and the receiver was changed periodically during the course of the extraction. Each fraction was evaporated to dryness under reduced pressure and weighed (Table XVII). It should be noted that these fractions were not completely free of solvent.

*Celite – a product of Johns-Manville.

TABLE XVI

YIELD OF HOT-WATER EXTRACTIVES

Tissue Layer	Weight of[a] Layer, g.	Vol. of Water Used for Extraction, l.	Time of Extraction, min.	Yield, g.	Total Yield, g.
Layer 1	45	1.0	35	2.85	
		1.0	30	1.01	
		1.0	50	0.08	3.94[b]
Layer 2	275	1.8	30	25.4	
		1.8	30	18.2	
		1.8	45	8.8	52.4[b]
Layer 3	855	5.7	45	64.0	
		5.7	60	26.0	
		5.7	70	14.6	94.6[b]
Layer 4	325	2.2	70	24.1	
		2.2	30	15.2	
		2.2	40	8.7	48.0[b]
Total	1500				198.9
Layer 1	45	1.2	30		4.32[c]
Layer 2	257	1.8	40		50.4[c]
Layer 3	869	5.7	60		99.5[c]
Layer 4	329	2.5	45		48.0[c]
Total	1500				202.2
Whole bark	1500	10.0	75		230.3[d]

[a] Variations due to differences in tissue layer separations.

[b] Based on three batch hot water extractions.

[c] Based on four batch hot water extractions.

[d] Based on four extractions with the volume and time indicated.

TABLE XVII

YIELD OF ETHYL ACETATE EXTRACTIVES

Tissue Layer	Time of Extraction, hr.	Yield[a], g.	Tissue Layer	Time of Extraction, hr.	Yield[b], g.
Layer 1	84	1.12	Layer 1	84	1.12
	264	0.36		192	0.20
	348	1.48		276	1.32
Layer 2	8	15.1	Layer 2	10	17.7
	18	5.4		178	12.2
	67	5.5		240	3.8
	96	3.6			
	168	2.4			
	349	32.0		428	33.7
Layer 3	9	19.1	Layer 3	9	30.5
	18	19.9		79	27.6
	73	11.7		330	14.2
	96	6.1			
	165	5.8			
	361	62.6		418	72.3
Layer 4	24	6.9	Layer 4	10	11.6
	92	7.4		284	9.1
	170	3.3		200	1.2
	286	17.6		494	21.9

Tissue Element	Time of Extraction, hr.	Yield, g.
Whole bark	10	20.5
	29	63.8
	120	35.2
	240	22.3
	168	6.1
	567	147.9
Condensate	65	5.3
	264	0.9
	329	6.2

[a] Based on first series of three batch hot water extractions.

[b] Based on second series of four batch hot water extractions.

ELUTION POLYAMIDE COLUMN CHROMATOGRAPHY

Further fractionation of the ethyl acetate-soluble hot water extractives was accomplished using polyamide column chromatography similar to that used in the past (15). A glass column 5 cm. in diameter and 120 cm. in length, fitted with a fritted glass filter and a stopcock at the base, was partially filled with distilled water. Polyamide powder* in the form of a water slurry was poured into the column, allowing the water to drain by gravity. Slurry was added in batches until the packed polyamide column height was 80 cm. The ethyl acetate extractives (20 to 40 g. from the fractions listed in Table VI) were dissolved in warm tetrahydrofuran and slurried with an equivalent amount of polyamide. When dry, this mixture was finely ground in a mortar and pestle.

The water level was lowered until it was two to three inches above the solid polyamide phase. In dry form the ethyl acetate extractive-polyamide mixture was added to the column which was then filled with distilled water. Elution was initiated and fractions (200 ml.) were collected at the rate of one every 40 minutes until a total of 120 was reached. All 200-ml. fractions were concentrated in a circulating evaporator, operating under reduced pressure, to about 5 ml. and set aside. Any crystalline materials found were filtered from the fraction, dried, weighed, and identified. Each fraction was then taken to dryness on a rotating vacuum evaporator and weighed to obtain individual fraction weights.

When water elution was completed, the polyamide was removed from the column and extracted, or elution was continued using a step-gradient elution technique, going from water to 95% ethanol. Polyamide removed from the column was extracted three times by boiling with water and filtering followed by triple extraction

*Polyamide Woelm, manufactured by M. Woelm, Eschwege, Germany.

with boiling ethanol. If elution was continued, another 200 fractions were collected and treated as indicated above. In certain instances the solids removed by extraction were slurried with polyamide and added to the top of freshly formed columns in preparation for gradient elution. In these cases columns were formed from polyamide powder slurried with 20% ethanol rather than with water. After eluting with ethanol (95%), the polyamide was removed and extracted three times with boiling ethanol.

Following evaporation to dryness, the weights of the water and ethanol extracts were noted for material balances. In most cases the extracted polyamide was allowed to dry and was employed in future work.

Since the extractives from Layer 1 and the condensate amounted to only a fraction of the other layers (1-3 g.) smaller columns and fraction volumes were used (Table XVIII).

TABLE XVIII

CONDITIONS FOR POLYAMIDE COLUMN CHROMATOGRAPHY OF LAYER 1 AND THE CONDENSATE

Fraction	Weight of Extractives, g.	Column Diameter, mm.	Packing Height, cm.	Eluate Volume, ml.
Layer 1	1.31	19	40	40
Condensate	2.93	23	80	50

CORRECTED ETHYL ACETATE EXTRACTIVE WEIGHTS

When the ethyl acetate extractives were dried on polyamide, a decreased weight usually was observed. Apparently, some solvent was entrained in the extractives and the increased surface area of the polyamide aided in its removal while drying (Table XIX). It should be noted that the extractives listed in Table XXIV (Appendix I) are not completely free of solvent.

TABLE XIX

CORRECTED WEIGHT OF ETHYL ACETATE EXTRACTIVES[a]

Fraction	Extractives Before Drying, g.	Polyamide, g.	Extractives After Drying, g.	Difference, %
Layer 1	1.48	3.75	1.31	13.0
Layer 2	32.0	30.4	30.3	5.6
Layer 3	40.0	39.8	36.4	9.9
Layer 4	17.6	31.7	17.6	0.0
Whole bark	40.0	40.5	35.4	13.0
Condensate	3.13	4.52	2.93	6.8

[a]From the ethyl acetate extractives in Table VI.

THIN-LAYER CHROMATOGRAPHY

Thin-layer chromatography was used to follow the variation from fraction to fraction and to aid in the preliminary identification of the various components eluted during polyamide column chromatography of the ethyl acetate extractives. Glass plates (10 x 20 cm.) were coated with a silica gel G* slurry using a Camag coater**. After air drying, the plates were activated by heating at 110°C. for one hour and then stored in a desiccator.

The prepared and spotted plates were developed by ascending chromatography in 4:1 chloroform-methanol (v/v), containing 5 ml. acetic acid per liter of solvent, for forty-five minutes to one hour. After developing, each plate was

*Silica gel G with gypsum binder. A O Inst. Comp., Analytical Inst. Div., 200 S. Garrard Blvd., Richmond, California.

**Manufactured by Camag, Switzerland.

checked for materials fluorescing under ultraviolet light, sprayed with 50% sulfuric acid in water (v/v), and heated in an oven at 95 to 110°C. for ten minutes. Component R_f's and colors were noted for each fraction. For data consistency, all R_f values were adjusted to that of salicin (R_f, 0.30). Unless otherwise specified, in this report thin-layer chromatography will refer to the techniques just described.

Appendix V correlated the R_f and colors observed for several known glucosides.

LEAD SUBACETATE TREATMENT OF THE WHOLE BARK ETHYL ACETATE EXTRACTIVES

Whole bark ethyl acetate extractives (43.5 g.) were diluted in 3000 ml. of distilled water. A slurry of 20 g. of fresh lead subacetate was added to this solution. After three hours at room temperature, another 20 g. of slurried lead subacetate were added to insure a suitable excess. The resulting precipitate was removed two hours later by centrifugation followed by filtration through a Celite pad. Hydrogen sulfide was bubbled through the initially pale green solution for 20 minutes after which the insoluble lead salts were filtered and washed, leaving a clear, colorless solution. To remove any excess hydrogen sulfide, the solution was boiled on a hot plate. Evaporation under reduced pressure yielded 16.2 g. of sirup.

Regeneration of the lead subacetate precipitate was accomplished by bubbling hydrogen sulfide through a two-liter suspension of the precipitate. After bubbling hydrogen sulfide through the solution for 35 minutes, the residual lead sulfide precipitate was removed by filtration, leaving a golden-yellow solution. Any residual hydrogen sulfide was eliminated by boiling on a hot plate. Evaporation

to dryness gave 15.2 g. of sirup. Extraction of the lead sulfite precipitate with tetrahydrofuran yielded another 3.1 g. of extractives. This scheme is reproduced in a flow chart in Fig. 24.

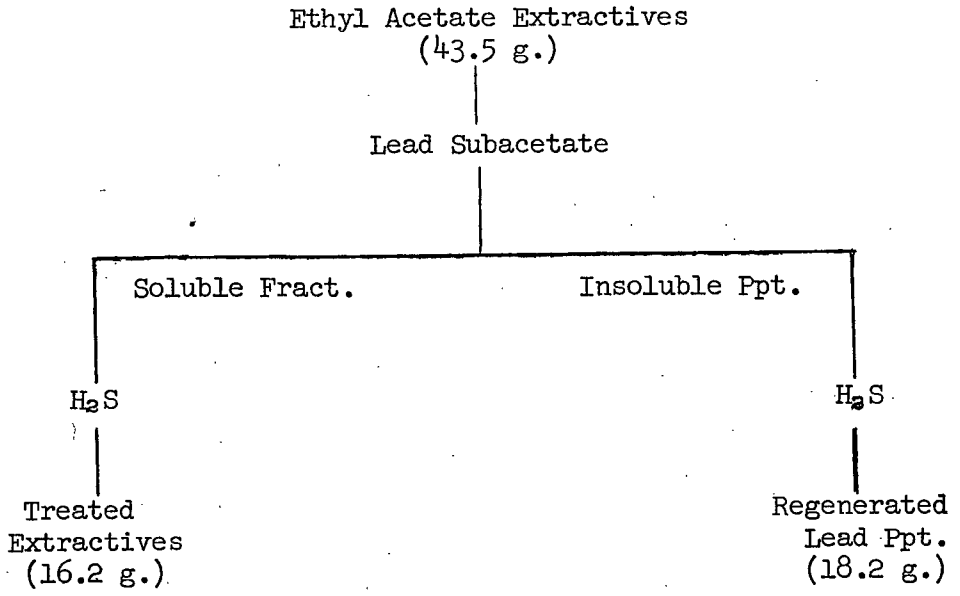


Figure 24. Flow Chart for the Lead Subacetate Treatment of the Whole Bark-1 Ethyl Acetate Extractives

Initially 43.5 g. of extractives were treated with lead subacetate; however, only 34.5 g. were recovered. Therefore, 9.0 g. (25%) must have remained with the lead precipitate.

QUANTITATIVE DETERMINATION OF SALICYL ALCOHOL AND PYROCATECHOL

Salicyl alcohol and pyrocatechol were shown to be present in the bark of P. grandidentata by thin-layer chromatography. Gas chromatography was used for the quantitative determination of these components in each of the tissue layers and in the whole bark ethyl acetate extractives after fractionation on a polyamide column. Chromatographic conditions are given in Tables XX and XXI.

TABLE XX

CONDITIONS FOR THE DETERMINATION OF SALICYL ALCOHOL BY GAS CHROMATOGRAPHY

Chromatograph	Aerograph Model A-100
Column	20% Apiezon L on Chrom W (60/80) SS
Column length	5 ft.
Column diameter	1/4 in.
Column temperature	150°C.
Gas flow rate	130 cc./min.

TABLE XXI

CONDITIONS FOR THE DETERMINATION OF PYROCATECHOL BY GAS CHROMATOGRAPHY

Chromatograph	Aerograph Model 200
Column	10% Apiezon N on Fluropak 80 SS
Column length	6 ft.
Column diameter	1/4 in.
Column temperature	220°C.
Gas flow rate	75 cc./min.
Retention time	3.0 min.

TOTAL YIELD OF 1-O-p-COUMAROYL-β-D-GLUCOSE

Polyamide column chromatography of the ethyl acetate extractives from the tissue layers and from the whole unsteamed bark indicated a considerable discrepancy in the yield of 1-O-p-coumaroyl-β-D-glucose. Whether degradation occurred or material had not crystallized from solution was not known.

The measurement by gas chromatography of 1-O-p-coumaroyl-β-D-glucose was not possible at the time of this study. Consequently, the 1-O-p-coumaroyl-β-D-glucose remaining in solution after crystallization of the fractions eluted on a polyamide column was saponified, so the p-coumaric acid could be analyzed as the trimethylsilyl (TMS) derivative. Paper chromatograms sprayed with DPNA indicated that 1-O-p-coumaroyl-β-D-glucose was the only p-coumaroyl derivative present in the fractions of interest.

An aliquot from each of the combined fractions under the 1-O-p-coumaroyl-β-D-glucose peaks (150-200 mg.) was removed and dried. Two ml. of 1% sodium hydroxide were added to each sample. After 15 minutes in a 60°C. hot water bath, these samples were neutralized and acidified with sulfuric acid. Each sample was extracted twice with ether; the extracts were dried on a rotary vacuum evaporator and then over phosphorus pentoxide.

Analysis for the TMS derivative of p-coumaric acid was made by gas chromatography using the Apiezon N column and chromatograph described in Table XXI. Each sample was trimethylsilylated by adding 150 µl. of dry pyridine followed by 150 µl. of Regisil*.

Known 1-O-p-coumaroyl-β-D-glucose was treated along with the unknown samples and analysis indicated that the saponification technique gave a value about 10% lower than theoretical when authentic p-coumaric acid was used as a control. The unknowns, however, were not corrected for this difference.

IDENTIFICATION OF POPULOSIDE

Populoside was found between Fractions 80 and 90 in Layer 3 and between 72 and 86 in the whole bark-2 ethyl acetate extractives during gradient elution with water and ethanol on a polyamide column. The former yielded about 230 mg. of populoside; the latter yielded more than 400 mg. Crude populoside was re-crystallized twice from water containing a small amount of ethanol to yield slightly yellow crystals melting at 186-188°C. and containing 2 1/2 moles of water. Populoside has a specific rotation of $[\alpha]^{27}_{Hg-546}$ -46.4° and $[\alpha]^{26}_{D}$ -38.0° (c, 2.23 in methanol).

*Regisil: Bis (trimethylsilyl) trifluoroacetamide, Regis Chemical Company, Chicago, Ill.

On thin-layer chromatography, populoside fluoresced under UV and exhibited a lavender streak having an R_f of 0.32. A faint yellow-brown streak was observed on the silica gel plate before spraying with sulfuric acid which corresponded with the populoside streak after spraying and heating. On a paper chromatogram developed in EPW and sprayed with DPNA, populoside exhibited a yellow-tan spot at an R_f of 0.75.

The infrared absorption curve (Fig. 36-Appendix VI) contained bands at 2.95, 3.47, 5.98, 6.12, 6.23, 6.59, 6.68, 6.90, 7.28, 7.86, 8.03, 8.38, 8.65, 9.00, 9.17, 9.30, 9.60, 10.22, 11.17, 11.62, 11.77, 12.40, and 13.20 μm.

An analysis for carbon and hydrogen corresponded with the proposed structure of populoside. Before analysis a sample was dried to a constant weight in an Abderhalden drying apparatus.

Calculated for $C_{22}H_{24}O_{10}$: C, 58.92; H, 5.36.

Found: C, 58.60, 58.54; H, 5.56, 5.61.

POPULOSIDE HEXAACETATE

Populoside (100 mg.) was acetylated with 1 ml. of acetic anhydride and 2 ml. of pyridine. After three hours in a water bath at 60°C. and 16 hours at room temperature, the acetylation mixture was added to 15 ml. of water and extracted three times with chloroform. The chloroform extract was concentrated and washed with 1N sulfuric acid, sodium bicarbonate, and water. The residual extract when concentrated to dryness yielded 165 mg. of colorless sirup. After three crystallizations from ethanol containing a little water, crystals of the hexaacetate were obtained which melted at 93-95°C. Some difficulty was encountered in obtaining crystals which gave a consistent melting point. The acetate had a specific rotation of $[\alpha]^{27}_{Hg-546}$ -9.33° and $[\alpha]^{26}_{D}$ -8.04° (c, 2.40 in chloroform).

The infrared absorption curve (Fig. 37 - Appendix VI) contained bands at 2.95, 3.42, 5.70, 5.82, 6.08, 6.20, 6.27, 6.65, 6.86, 7.00, 7.30, 7.55 μm., and a broad band centered at 8.20, 8.51, 9.00, 9.37, 9.61, 9.88, 10.17, 11.05, 11.40, 11.98, 13.22, and 14.38 μm.

Drying of the acetate at 100°C. in a vacuum always decreased the weight of solids, indicating that some solvent remained associated with the hexa-acetate crystals. Analyses for carbon and hydrogen after drying corresponded with the proposed structure of populoside hexaacetate.

Calculated for $C_{34}H_{36}O_{14}$: C, 58.29; H, 5.14.

Found: C, 58.22, 57.93, 57.94; H, 5.24, 5.18, 5.17.

Loss at 100°C., %: 2.57, 2.36, 2.38.

The molecular weight determined by mass spectrometry was 700.

ENZYMATIC HYDROLYSIS

Enzymatic hydrolysis of populoside with beta-glucosidase* indicated that glucose was liberated. Ninety-six mg. of populoside were added to 50 ml. of 0.1N sodium acetate buffered to a pH of 5. After heating to dissolve the crystals, the solution was cooled and maintained at a temperature of 30 to 40°C. About 50 mg. of beta-glucosidase were added to the buffered solution. After three hours, the aqueous solution was extracted with ether. This extract was dried overnight with sodium sulfate and concentrated to give an aglucone which on paper gave a brick-red spot at an R_f of 0.82 when developed in EPW and sprayed with DPNA.

The aqueous phase was passed through an acid-regenerated Amberlite IR-120** ion-exchange column and concentrated. Thin-layer chromatography indicated the presence of glucose when a silica gel chromatogram was developed in 2:1 ethyl acetate-chloroform (v/v), sprayed with sulfuric acid, and charred on a hot plate.

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*Obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio.

**A product of Rohm and Haas Company.

ALKALINE HYDROLYSIS OF POPULOSIDE : BARIUM HYDROXIDE

Since the infrared spectrum of populoside indicated an ester group, 100 mg. were saponified in 20 ml. of saturated barium hydroxide and 5 ml. of water. All of the glucoside would not go into solution. Heating turned the solution a very dark reddish-brown with some solids present. After boiling between three and four minutes, the sample was cooled and filtered through a sintered glass filter to yield a clear but dark solution. A few mg. of residue were retained by the filter. Dilute sulfuric acid was used to neutralize the solution to a Congo red end point and the resulting barium sulfate was removed by filtration. The filtrate was extracted three times with ethyl ether, which upon concentration gave 23 mg. of crude material melting at 212-213°C. with decomposition. Recrystallization from water yielded dark crystals melting at 214-215°C. with decomposition.

Paper chromatograms of the organic acid obtained from the alkaline saponification of populoside and caffeic acid were identical. Both gave a yellow-brown spot at an R_f of 0.75 when developed in EPW and sprayed with DPNA. The former exhibited a slight purple fringe at the leading edge of the caffeic acid spot. This has been attributed to the presence of p-coumaric acid which may have come from trichocarposide (6-O-p-coumaroyl salicin). Populoside and trichocarposide have been shown to be eluted concurrently from a polyamide column (40).

To remove any excess sulfuric acid the slightly acidic raffinate was treated with excess barium carbonate and filtered. Concentrating the aqueous solution to dryness gave 64 mg. of crude salicin. This was taken up in a 50% ethanol solution and centrifuged to eliminate a residual precipitate. The clear supernatant liquid was evaporated to yield 45 mg. of relatively pure salicin

melting at 198-199°C. and not depressing a mixed melting point with authentic salicin. Thin-layer chromatography for the unknown was the same as for salicin.

ALKALINE HYDROLYSIS OF POPULOSIDE : SODIUM HYDROXIDE

It was assumed that degradation was occurring when populoside was saponified with barium hydroxide because the ether extract was colored. Consequently, 40 mg. of populoside were hydrolyzed with 10% sodium hydroxide. All of the populoside dissolved giving a golden-yellow solution. After five to six minutes, the reaction mixture was neutralized with dilute sulfuric acid and then slightly acidified. Sodium sulfate crystals came out of solution on cooling. Following filtration, the aqueous solution was extracted with ethyl ether which upon concentration yielded 12 mg. of a light brown material. This was recrystallized from an ethanol-water solution to give fine yellow-brown crystals melting at 213-215°C. with decomposition. An IR spectrum (Fig. 41-Appendix VI) was found to be identical with that of caffeic acid.

Samples of the unknown organic acid and authentic caffeic acid had the same gas chromatographic retention times when chromatographed as the TMS derivative (Table XXII).

TABLE XXII

CONDITIONS FOR THE GAS CHROMATOGRAPHIC DETERMINATION OF CAFFEIC ACID AS THE TMS DERIVATIVE

Chromatograph	Aerograph Model 200
Column	5% SE 30 on Anakrom ABS (60/70) SS
Column length	5 ft.
Column diameter	1/4 in.
Column temperature	220°C.
Gas flow rate	75 cc./min.
Retention time	6.4 min.

IDENTIFICATION OF GRANDIDENTOSIDE

When ethyl acetate extractive samples were subjected to elution chromatography with water on a polyamide column, grandidentoside was isolated in combination with grandidentatin between Fractions 60 and 80. Separation of the mixture was accomplished by silica gel* column chromatography. A column (1 x 70 cm) was packed to a height of 57 cm. with a slurry of silica gel in 93% chloroform, 5% methanol, and 2% formic acid. Fifty-three mg. of the mixture were taken up in the same solvent and pipetted onto the column. Elution was initiated and 20-ml. fractions were collected. However, the methanol concentration was increased in increments to induce a gradient. A separation of the components was achieved as indicated in Fig. 25, yielding 31 mg. of grandidentoside.

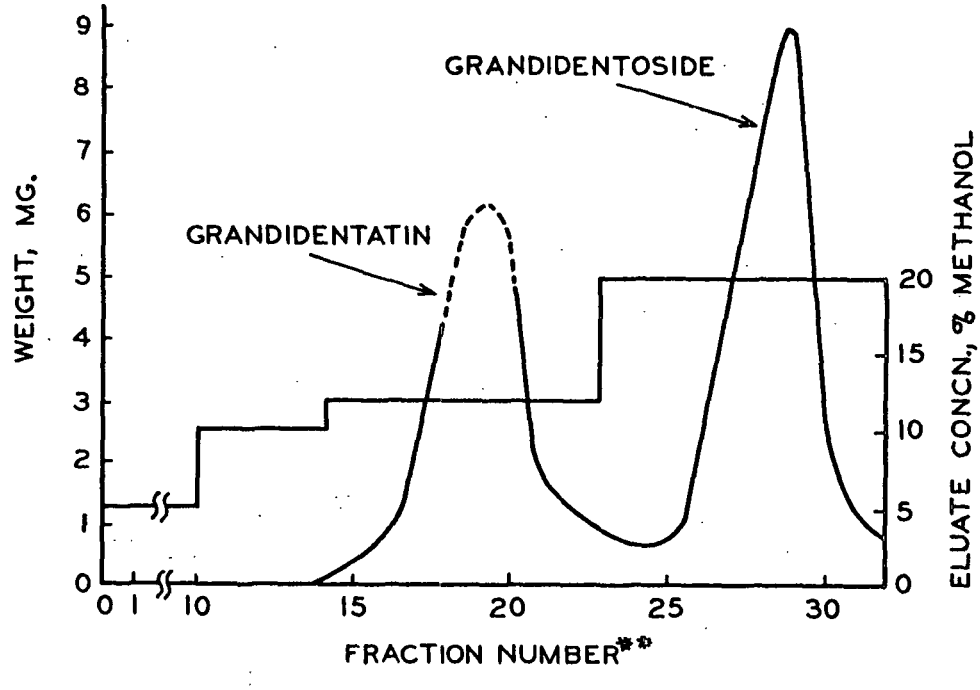

Figure 25. Silica Gel Column Separation of a Grandidentatin and Grandidentoside Mixture. Weight of Solids Applied to the Column: 53 mg.

*Silica gel: 60-200 mesh, Grade 950; Fisher Scientific Company, Fair Lawn, New Jersey.

**Fraction 19 was partially lost, so the dotted line was used to complete the elution curve.

Grandidentoside crystals were obtained by crystallization of 20 mg. of crude sirup in about 1 ml. of water containing some methanol and formic acid. Once the sirup was completely in solution, the latter two solvents were partially removed on a rotary vacuum evaporator. After two days in the refrigerator, the solution deposited 3.5 mg. of white clustered needles melting at 196-197°C.

A silica gel thin-layer chromatogram of grandidentoside exhibited a lavender streak having an $\underline{R}_f$ of 0.35. The use of formic acid in the developing solvent eliminated streaking. A faint yellow-brown streak was observed before spraying. This corresponded with the grandidentoside streak which appeared after spraying with sulfuric acid and heating. Paper chromatograms developed in 1:1:1 butanol-ethanol-water and sprayed with DPNA exhibited a yellow-tan spot at an $\underline{R}_f$ of 0.81.

The infrared absorption curve (Fig. 39-Appendix VI) contained bands at 2.97, 3.16, 3.45, 5.85, 6.14, 6.21, 6.61, 6.92, 7.28, 7.48, 7.76, 8.00, 8.46, 8.65, 8.97, 9.30, 9.40, 9.68, 10.01, 10.20, 10.50, 10.94, 11.22, 11.75, 12.26, 12.73, and 13.03 μm.

GRANDIDENTOSIDE HEXAACETATE

Grandidentoside sirup (9.5 mg.) was acetylated with 1 ml. of acetic anhydride and 2 ml. of pyridine. After 16 hours, the acetylated mixture was worked up as described for populoside hexaacetate to give 13 mg. of grandidentoside hexaacetate sirup. This was taken up in about 0.7 ml. of 95% ethanol to which a few drops of water were added. Twenty-four hours later, 3.3 mg. of fine needles melting at 175-177°C. were removed.

The infrared absorption curve (Fig. 40-Appendix VI) contained bands at 2.93, 3.40, 3.50, 5.72, a broad band centered at 5.72, 6.11, 6.67, 7.01, 7.30, 8.06, 8.29, 8.49, 9.00, 9.47, 9.67, 10.16, 10.45, 11.11, 12.00, and 16.68 μm.

The molecular weight determined by mass spectrometry was 692.

ALKALINE HYDROLYSIS OF GRANDIDENTOSIDE

Five ml. of water and 1 ml. of clear, saturated barium hydroxide were added to 38.1 mg. of slightly colored grandidentoside. In a short time the solution turned a dark brown. After 10 minutes at room temperature, warming on a steam bath produced a darker solution exhibiting a fine precipitate. Centrifugation left a clear yellow-brown solution which was subsequently neutralized with 1N sulfuric acid. Following removal of the barium sulfate precipitate by centrifugation, the supernatant liquid was passed through an Amberlite MB-3* ion-exchange column. The effluent from the column was evaporated to dryness yielding 14.5 mg. of chromatographically pure colorless sirup.

About 2 mg. of pure grandidentoside sirup were saponified in 1/2 ml. of clear barium hydroxide and 1/2 ml. of water. After 15 minutes at room temperature, the solution was acidified with 1N sulfuric acid. The acidified mixture was filtered and extracted with ether. A thin-layer chromatogram of the ether extract exhibited a single pink-lavender spot at an R_f of 0.55 which corresponded with authentic caffeic acid.

ACETYLATION OF THE DECAFFEOYLATED MOIETY

Acetylation was accomplished by adding 1 ml. of acetic anhydride and 2 ml. of pyridine to 14.5 mg. of deesterified grandidentoside sirup. After 24

*Amberlite MB-3: A product of Rohm and Haas Company.

hours, the acetylation mixture was worked up as described for populoside hexaacetate to yield 25.6 mg. of colorless sirup. This was transferred to a 10-ml. Erlenmeyer flask, and 1 ml. of 95% ethanol and 2 ml. of water were added. Fine needlelike crystals appeared after two hours of refrigeration. Filtering yielded 15.0 mg. of crystals melting at 120-121°C. which did not depress a mixed melting point with authentic grandidentin pentaacetate. An infrared absorption spectrum was also identical with known grandidentin acetate (Fig. 41-Appendix VI).

SILICA GEL COLUMN SEPARATION OF A COMPLEX EXTRACTIVE MIXTURE

Since separation of grandidentatin and grandentoside was successful by silica gel column chromatography, an attempt to separate a more complex mixture was made. A column 1 cm. in diameter was packed to a height of 57 cm. using a slurry of silica gel in a solvent composed of 93% chloroform, 5% methanol, and 2% formic acid. Extractives (150 mg.), from Fractions 61 to 85 of the whole bark ethyl acetate extractives after polyamide column chromatography, were pipetted onto the silica gel column in the above solvent. Although elution was initiated with this solvent, the methanol concentration was increased in increments. After the elution of 51 fractions (20 ml.) all of the extractives initially placed on the column had been eluted. The elution curve may be found in Fig. 22.

CARBON AND HYDROGEN ANALYSES

All carbon and hydrogen analyses were performed by Micro-Tech Laboratories, Inc., 4117 Oakton Street, Skokie, Illinois.

INFRARED SPECTRA

Infrared spectra were obtained with a Perkin-Elmer, Model 21 prism infrared spectrophotometer or a Perkin-Elmer, Model 621 grating infrared spectrophotometer. The former measured transmittance and was linear in wavelength (μm.); the latter measured absorbance and was linear in frequency ($cm.^{-1}$) with a 2:1 scale change between 2000 and 2500 $cm.^{-1}$ A sodium chloride prism was used in the Model 21 and in each case, potassium bromide pellets were prepared.

MASS SPECTRA

Mass spectra were determined by Morgan-Schaffer Corp., Montreal, Quebec, Canada, using a double-focusing, Hitachi RMU-6D instrument by direct introduction of the sample in the ionizing beam.

MELTING POINTS

Melting points were determined with a Thomas Hoover capillary melting point apparatus from Arthur H. Thomas Company, Philadelphia, Pa. All melting points reported are uncorrected.

NUCLEAR MAGNETIC RESONANCE SPECTRA

Nuclear magnetic resonance spectra were determined on a Varian Model A-60 A analytical spectrometer using methyl sulfoxide-d_6* as the solvent. Exchange of the hydroxyl groups was accomplished using deuterium oxide**. Tetramethylsilane*** was used as a reference source.

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*Methyl sulfoxide-d_6: Stohler Isotope Chemicals, 92 Beckwith Pl., Rutherford, New Jersey.

**Deuterium oxide: Stohler Isotope Chemicals, 92 Beckwith Pl., Rutherford, New Jersey.

***Tetramethylsilane: Aldrich Chemical Co., Inc., Milwaukee, Wisconsin.

SUMMARY AND CONCLUSIONS

The smooth green bark of Populus grandidentata was separated into distinct anatomical layers after a brief period of steaming. A general analysis of the total extractives, however, indicated that separation in this manner modified the nature of some of the resulting components.

Fractionation of the ethyl acetate-soluble hot water extractives from each layer and from the whole unsteamed bark by elution polyamide column chromatography yielded nine crystalline glucosides. These included salicin, salicortin, 1-O-p-coumaroyl-β-D-glucose, populin, tremuloidin, grandidentatin, grandidentoside, salireposide, and populoside. Grandidentoside and populoside were both new glucosides which were characterized in the present study. All of these components, isolated from the tissue layers, also were obtained from the whole bark. This observation led to the conclusion that there was no advantage in using the separate tissue layers rather than the whole bark in the analysis of the hot water extractives from this species.

In all cases the yields of individual crystalline components from the tissue layers were lower than those from the whole unsteamed bark ethyl acetate extractives. A total crystalline yield from all layers only amounted to 9.4 g. compared with over 20 g. (from 1500 g. o.d. bark) isolated from the whole bark. Specific analysis for 1-O-p-coumaroyl-β-D-glucose indicated that only 40% of the total present in the whole bark could be accounted for in the tissue layers. Apparently, separation of the bark into its anatomical fractions by the steaming process promoted degradation of certain components. Degradation may have arisen from either the high temperatures and/or from oxidation as the moist tissue layers were exposed to the air. Caution should be used in any future work where steaming is employed. An alternate method, such as freeze drying, prior to hand

classification of the whole bark probably is the best approach. Application of freeze drying in an analysis of the whole bark extractives might also reduce any phenomenon of degradation.

Separation of P. grandidentata bark into its anatomical fractions did not yield any new components. However, separation showed that the identified components were not restricted entirely to the cambial zone or to any other specific region. A higher concentration of both salicin and salicortin was detected in the inner bark. Seventy percent of the salicyl alcohol present in the whole bark was found in the outer region (Layer 1). Pyrocatechol was most concentrated in the inner bark (Layer 4); only traces were found in the outer bark (Layer 1). It was not possible, however, to determine a gradient of components over the whole bark.

A lead subacetate treatment, commonly used for the removal of interfering materials, was shown to modify seriously the ethyl acetate-soluble hot water extractives. Essentially no tremuloidin was found in the untreated extractives, but substantial yields of this component were recovered after lead subacetate treatment. This treatment also eliminated salicortin, grandidentoside, and pyrocatechol. Decreased yields of 1-O-p-coumaroyl-β-D-glucose, grandidentatin, and salireposide were observed; a slightly increased amount of salicin was noticed.

Gradient elution (aqueous ethanol solvent) of the ethyl acetate extractives on a polyamide column after elution with water proved to be extremely successful in fractionating a rather significant proportion of the extractives. This technique led to the isolation of populoside.

Although a major separation of the ethyl acetate extractives was accomplished by elution polyamide column chromatography, the eluate fractions were still quite complex. Silica gel column chromatography in conjunction with a gradient elution technique proved to be an effective means for further separating these complex fractions.

Nuclear magnetic resonance spectrometry was used in determining the location of the caffeoyl group on glucose of grandidentoside. An analysis of phenolic glucosides with established structures indicated that this analytical tool can be used for locating substituents on the carbohydrate moiety with great facility. Its use is especially warranted when only small quantities of material exist, and the nature of the aglucone makes reactions such as methylation impractical.

SUGGESTIONS FOR FUTURE WORK

To enlarge and extend the fundamental concepts of this thesis, the following suggestions for future studies are set forth:

1. More comprehensive analysis of the primary phenolic glucosides as a function of the morphological composition of the bark. Since steaming of the bark resulted in the degradation of certain components, freeze drying of fresh whole bark prior to separation into tissue layers should yield fractions with an unchanged composition. Gas chromatography would be the best technique quantitatively determining each component. However, preliminary fractionation by polyamide column chromatography (small scale) is necessary to reduce the complexity of the extractive mixture.

2. Application of large-scale silica gel column chromatography to the fractions after polyamide chromatography as applied on a small scale in this thesis (p. 66). This technique should allow for the isolation of many more phenolic glucosides, the presence of which is indicated by thin-layer chromatography.

3. Synthesis of identified components found in this study and related derivatives. Since there are several likely positions of substitution on salicin, grandidentin, etc., synthesis of the various cinnamoyl ester derivatives, among others, with subsequent comparison with extractive components is an alternate route to the identification of minor constituents.

GLOSSARY

Chromatography

EPW	= 8:2:1 ethyl acetate – pyridine – water
DPNA	= diazotized *p*-nitroaniline followed by sodium carbonate
R_f	= ratio with solvent front
R_G	= ratio with glucose
Thin-layer	= silica gel plates developed in 4:1 chloroform-methanol (trace of acetic acid), sprayed with 50% sulfuric acid, and heated in an oven at 105°C. for 10 min.
TMS	= trimethylsilylated

General

Layer 1	= phellem
Layer 2	= phelloderm
Layer 3	= outer portion of secondary phloem
Layer 4	= inner portion of secondary phloem
Whole bark-1	= first fractionation of the whole bark ethyl acetate extractives by polyamide column chromatography
Whole bark-2	= second fractionation of the whole bark ethyl acetate extractives by polyamide column chromatography
IR	= infrared
UV	= ultraviolet
m/e	= mass-to-charge ratio
DMSO	= methyl sulfoxide

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The valuable assistance of the following is also acknowledged with gratitude: Dr. D. C. Johnson for guidance in NMR interpretations; Mr. L. O. Sell for instruction in the use of the NMR spectrometer and for determining infrared absorption spectra; Mr. J. D. Hankey for preparing bark photomicrographs; Mr. M. K. Benson for assistance in obtaining fresh P. grandidentata trees; and the Analytical Department of The Institute of Paper Chemistry. Above all, I am indebted to my wife, Nancy, for her continual encouragement and for her help in preparing the original manuscript of this dissertation.

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APPENDIX I

HOT WATER AND ETHYL ACETATE EXTRACTION DATA FOR *POPULUS GRANDIDENTATA* WHOLE BARK

Table XXIII corresponds with the second series of hot water extractions involving four repetitions of the batch extraction process. Table XXIV presents the subsequent ethyl acetate extraction data for these hot water extractions.

TABLE XXIII

DISTRIBUTION OF HOT WATER EXTRACTIVES IN POPULUS GRANDIDENTATA BARK

Tissue Layer	Weight of Tissue[a] Layer, g.	Hot Water Extractives Yield, g.	Tissue Layer Yield, %	Whole Bark Yield, %
Layer 1[b]	45	4.32	9.60	0.29
Layer 2[b]	257	50.4	19.6	3.36
Layer 3[b]	869	99.5	11.5	6.63
Layer 4[b]	329	48.0	14.6	3.20
Total	1500	202.2		13.48

[a]Based on the contribution of each layer to the ovendry weight of 1500 g. of whole bark. Minor differences when compared with Table V are due to tissue separation variations.

[b]Based on four batch hot water extractions.

TABLE XXIV

DISTRIBUTION OF ETHYL ACETATE SOLUBLE-HOT WATER EXTRACTIVES IN POPULUS GRANDIDENTATA BARK

Tissue Layer	Period of Extraction, hr.	Ethyl Acetate[a] Extractives Yield, g.	Hot Water Extract Yield, %	Tissue Layer Yield, %	Whole Bark Yield, %
Layer 1[b]	280	1.48	34.3	3.29	0.10
Layer 2[b]	430	33.7	66.8	13.1	2.25
Layer 3[b]	420	72.3	72.7	8.33	4.85
Layer 4[b]	490	21.8	45.4	6.63	1.45
Total		129.3			8.65

[a] Extractives not completely free of solvent.

[b] Based on second series of four batch hot water extractions.

APPENDIX II

POLYAMIDE COLUMN CHROMATOGRAPHY ELUTION CHROMATOGRAMS

Figure 26 illustrates the water-eluted polyamide column chromatogram for the whole bark-1 ethyl acetate extractives. Figures 27 and 28 show the gradient elution polyamide column chromatograms for Layers 2 and 3, respectively. Gradient elution was started immediately after water elution was discontinued, i.e., the solids were not removed and placed on freshly packed columns.

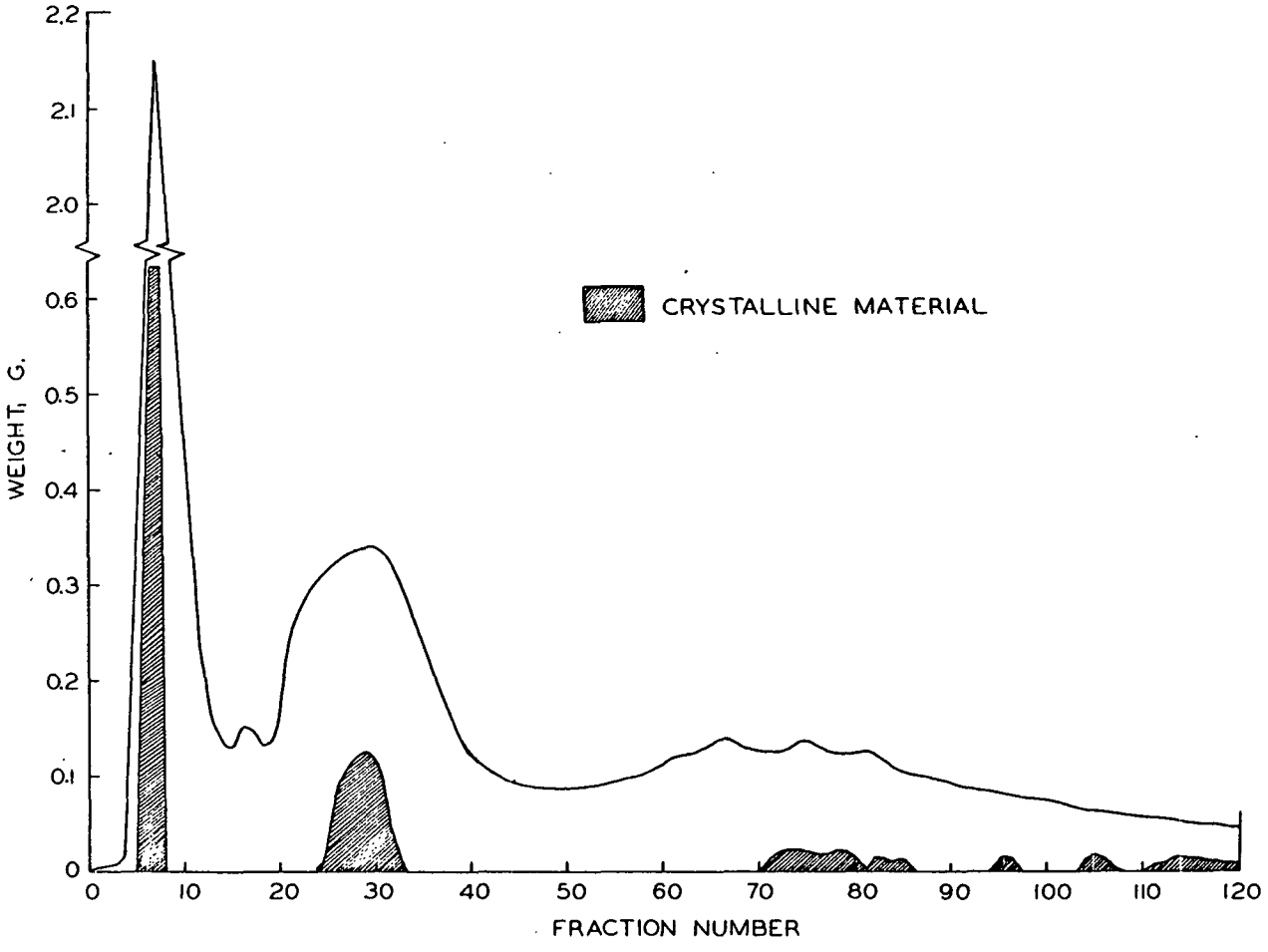

Figure 26. Polyamide Column Chromatography of the Ethyl Acetate Extractives from Populus grandidentata Whole Bark-1. Weight of Extractives Applied to the Column: 35.4 g.

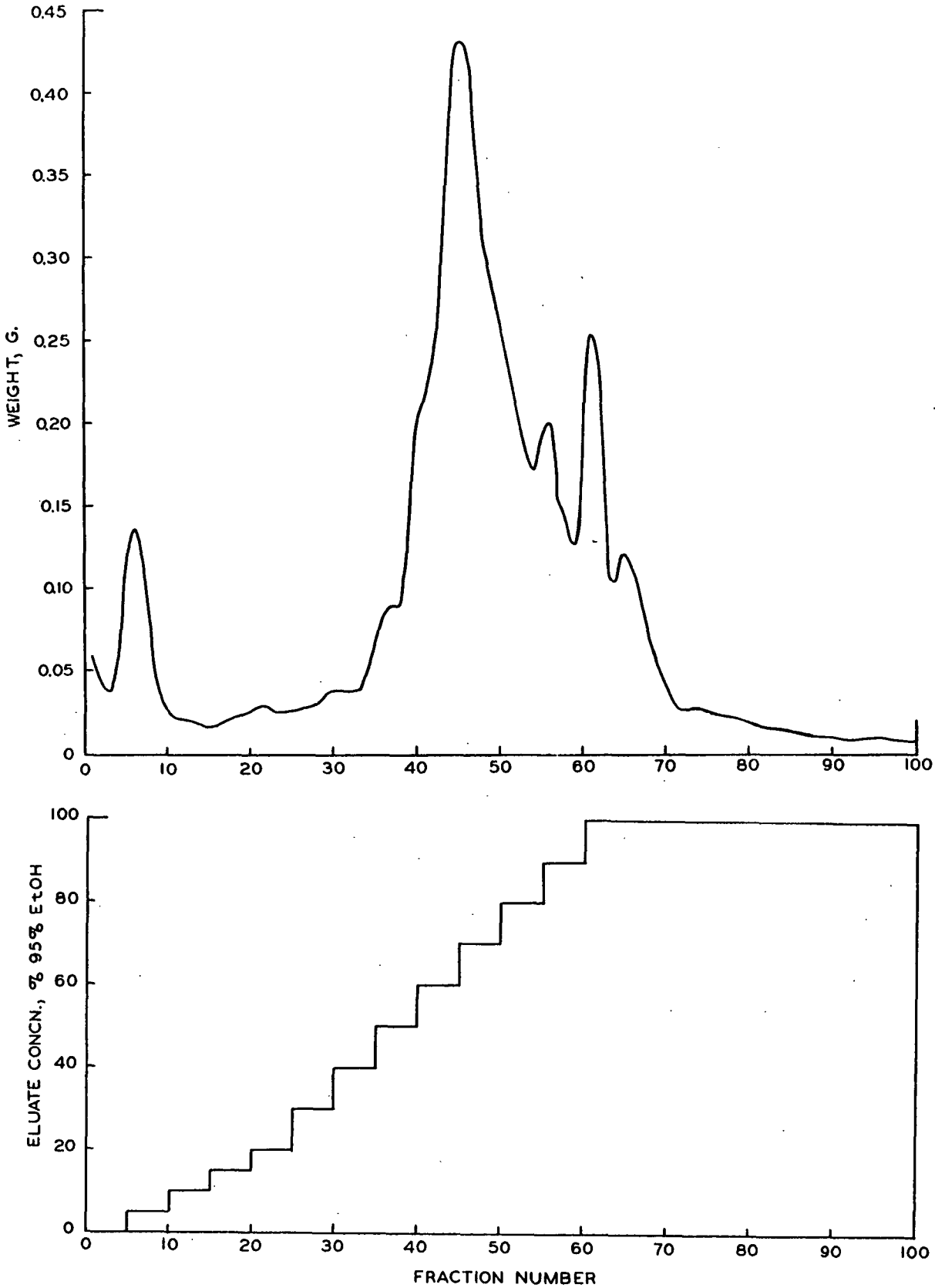

Figure 27. Gradient Elution Polyamide Column Chromatography of the Ethyl Acetate Extractives from Populus grandidentata Bark Layer-2. Weight of Extractives Applied to the Column: 10.6 g.

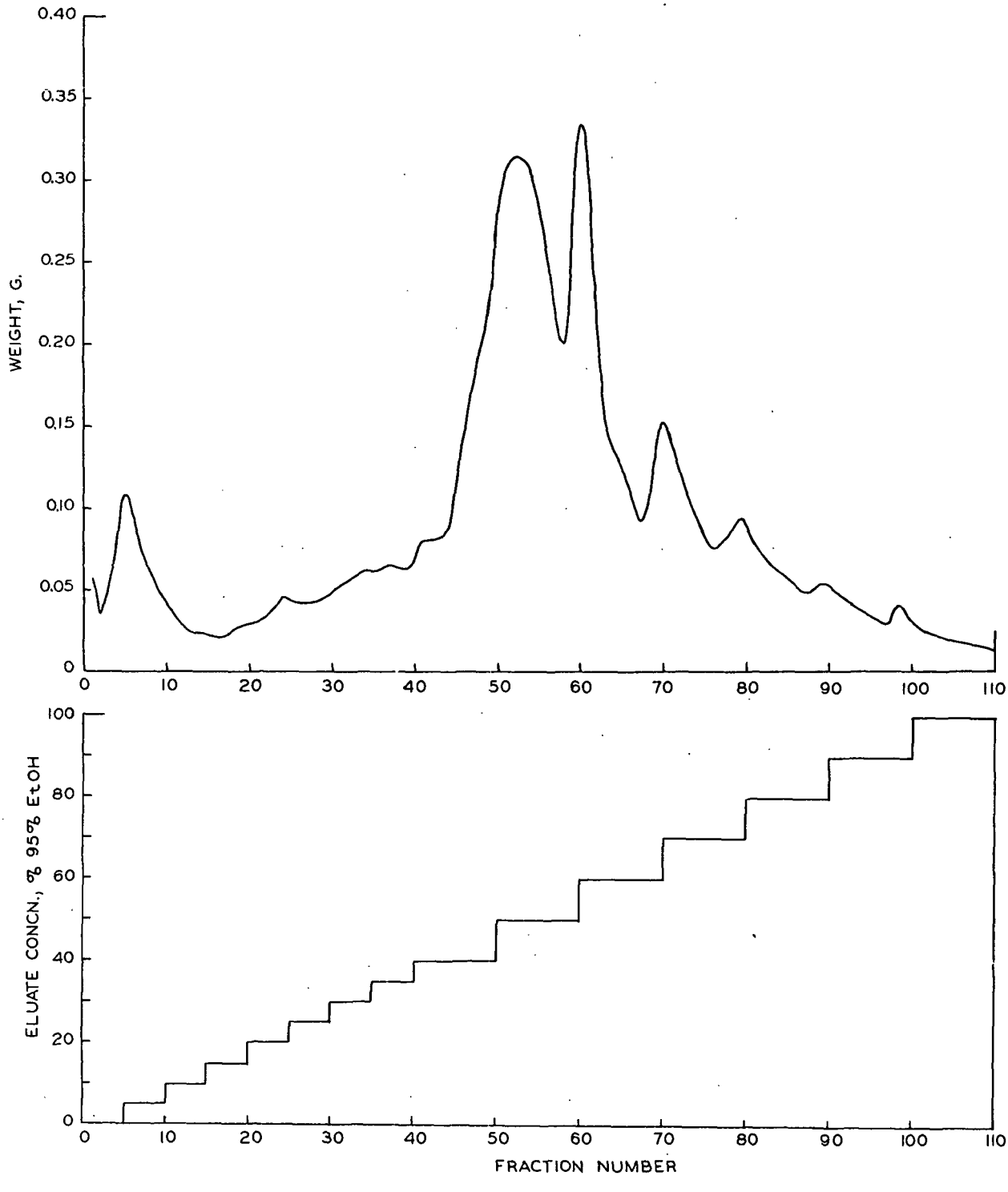

Figure 28. Gradient Elution Polyamide Column Chromatography of the Ethyl Acetate Extractives from Populus grandidentata Bark Layer-3. Weight of Extractives Applied to the Column: 12.9 g.

APPENDIX III

MASS SPECTRAL DATA FOR POPULOSIDE AND GRANDIDENTOSIDE HEXAACETATES

TABLE XXV

MASS-TO-CHARGE RATIO VERSUS RELATIVE INTENSITY FOR PEAKS FROM THE MASS SPECTRUM OF POPULOSIDE HEXAACETATE

m/e	Relative Intensity, %	m/e	Relative Intensity, %
43	100.0	205	5.0
69	2.4	211	3.7
77	2.4	229	2.7
78	3.7	247	1.3
81	6.4	271	5.0
85	3.4	289	4.0
97	6.1	328	5.0
103	4.7	331	21.5
107	12.5	332	3.7
109	55.5	345	0.1
110	5.1	370	0.2
115	3.4	412	0.03
127	17.5	437	0.01
134	4.0	535	0.007
135	4.0	556	0.007
139	6.4	572	0.01
145	6.7	599	0.003
149	2.0	616	0.02
162	2.7	627	0.007
163	12.8	641	0.04
169	86.9	658	0.03
170	8.7	700	0.05
180	3.7		

TABLE XXVI

MASS-TO-CHARGE RATIO VERSUS RELATIVE INTENSITY FOR PEAKS FROM THE MASS SPECTRUM OF GRANDIDENTOSIDE HEXAACETATE

m/e	Rel. Intensity, %	m/e	Rel. Intensity, %	m/e	Rel. Intensity, %
28	16.4	163	89.4	428	0.4
42	11.8	164	11.0	450	10.2
43	100.0	168	3.9	451	3.0
55	3.1	169	15.9	452	0.7
60	2.0	M 170	247 → 205 + 42	464	0.2
61	2.9	180	3.7	468	0.2
69	4.1	187	3.1	492	5.2
70	7.6	189	10.6	493	1.7
71	2.9	200	2.4	510	0.6
73	3.5	204	5.7	535	0.4
81	31.7	205	92.0	536	0.1
85	5.7	206	13.9	548	0.03
97	11.4	219	5.1	549	0.01
98	10.8	222	1.6	550	0.01
99	27.2	228	8.4	552	0.5
100	2.5	229	3.3	553	0.01
103	2.7	247	33.6	M 569	650 → 608 + 42
109	11.2	248	5.7	577	0.02
110	2.9	259	1.0	590	0.02
115	11.9	264	1.2	591	0.01
126	3.7	271	1.0	592	0.01
127	5.9	288	1.0	608	5.0
M 129.5		295	1.8	609	0.2
133	3.1	331	0.6	619	0.04
134	12.6	348	0.7	633	0.02
135	6.1	362	0.5	650	0.5
136	2.5	368	1.3	664	0.06
139	8.0	369	0.7	692	0.03
140	4.1	390	0.8		
141	22.9	391	0.2		
142	2.2	404	0.2		
145	5.1	408	0.6		
147	4.9	409	0.1		
155	4.1	M 412	492 → 450 + 42		
157	5.1	422	0.6		
162	63.7	423	0.1		

APPENDIX IV

NUCLEAR MAGNETIC RESONANCE SPECTRA

Figures 29 - 35 illustrate the partial NMR spectra of salicin, salicyloyl salicin, tremuloidin, populin, grandidentin, grandidentatin, and grandidentoside. These spectra were used to aid in the location of the caffeoyl group on the glucose moiety of grandidentoside.

Figure 36 portrays the entire spectrum of populoside.

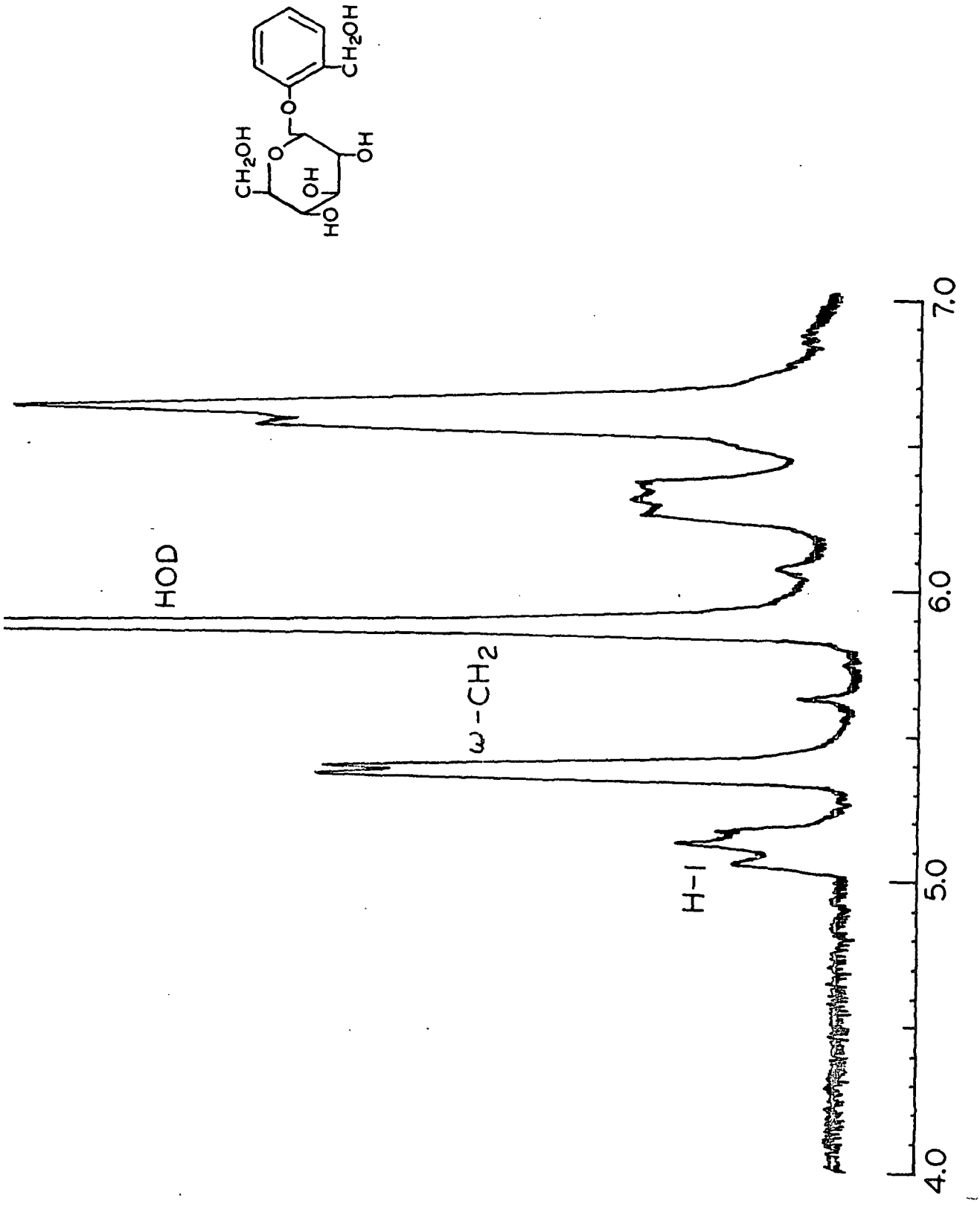

Figure 29. NMR Spectrum of Salicin (60 MHz) in Methyl Sulfoxide-d_6 Containing Deuterium Oxide

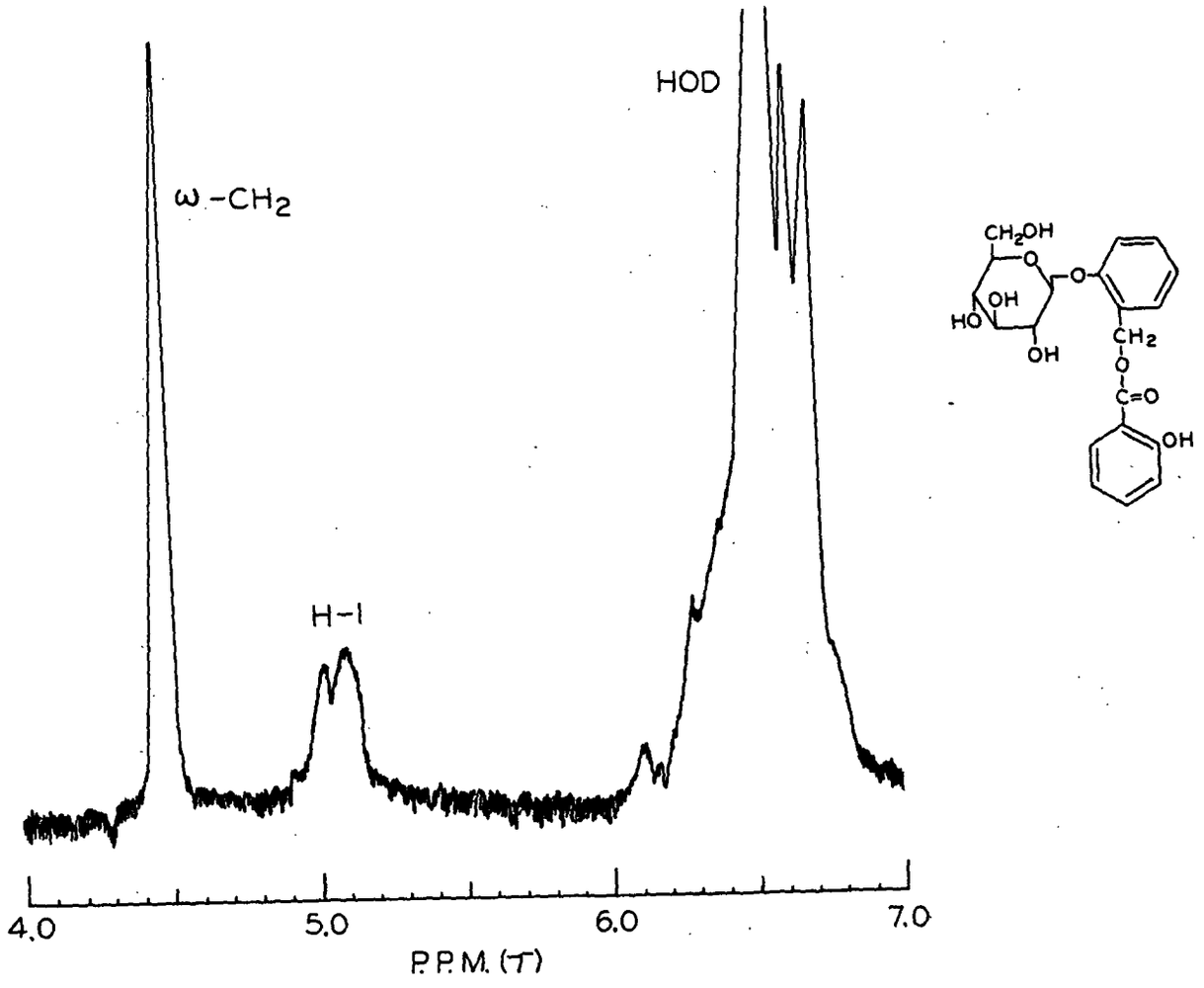

Figure 30. NMR Spectrum of Salicyloyl Salicin (60 MHz) in Methyl Sulfoxide-d_6 Containing Deuterium Oxide

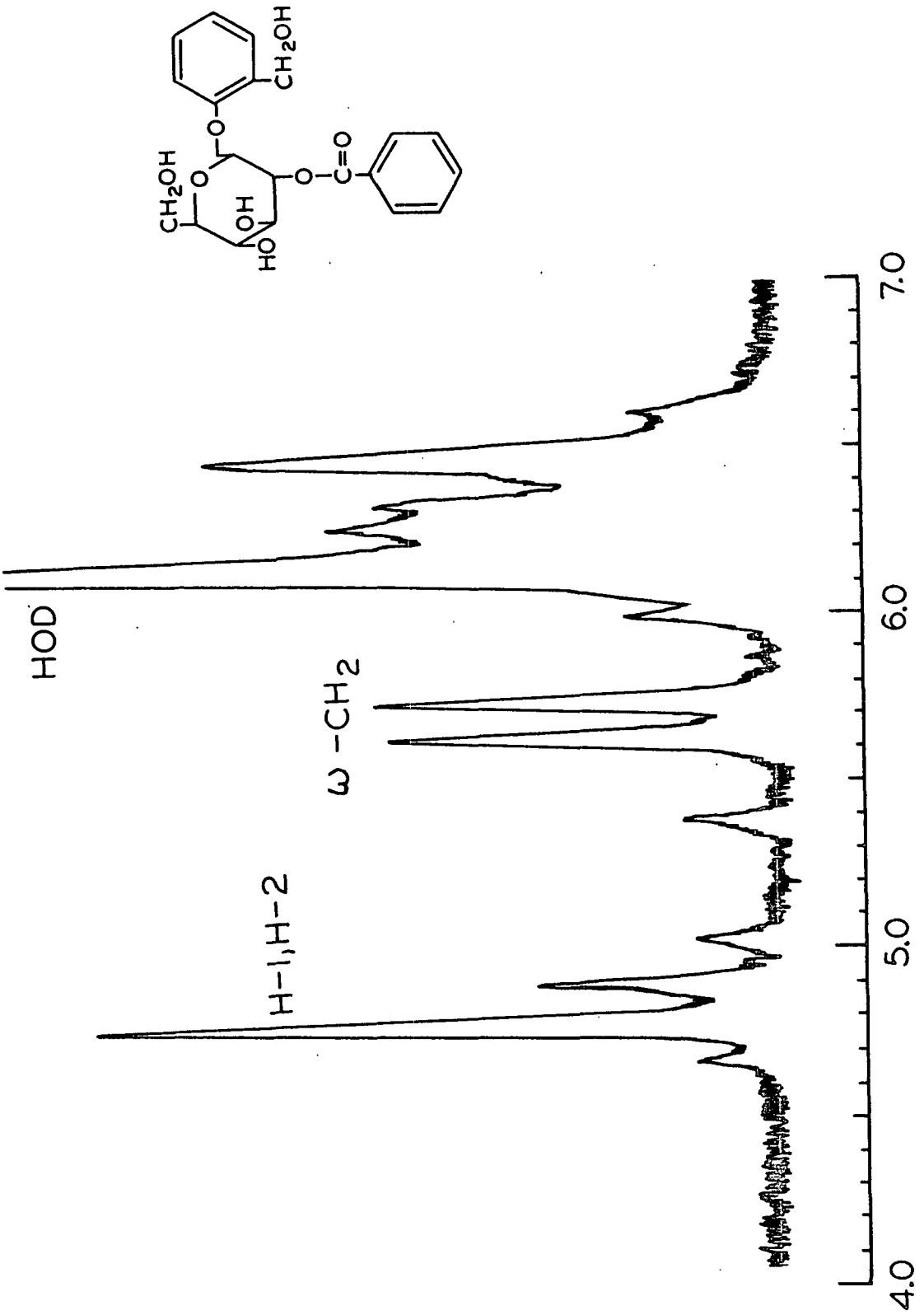

Figure 31. NMR Spectrum of Tremuloidin (60 HMz) in Methyl Sulfoxide-d_6 Containing Deuterium Oxide

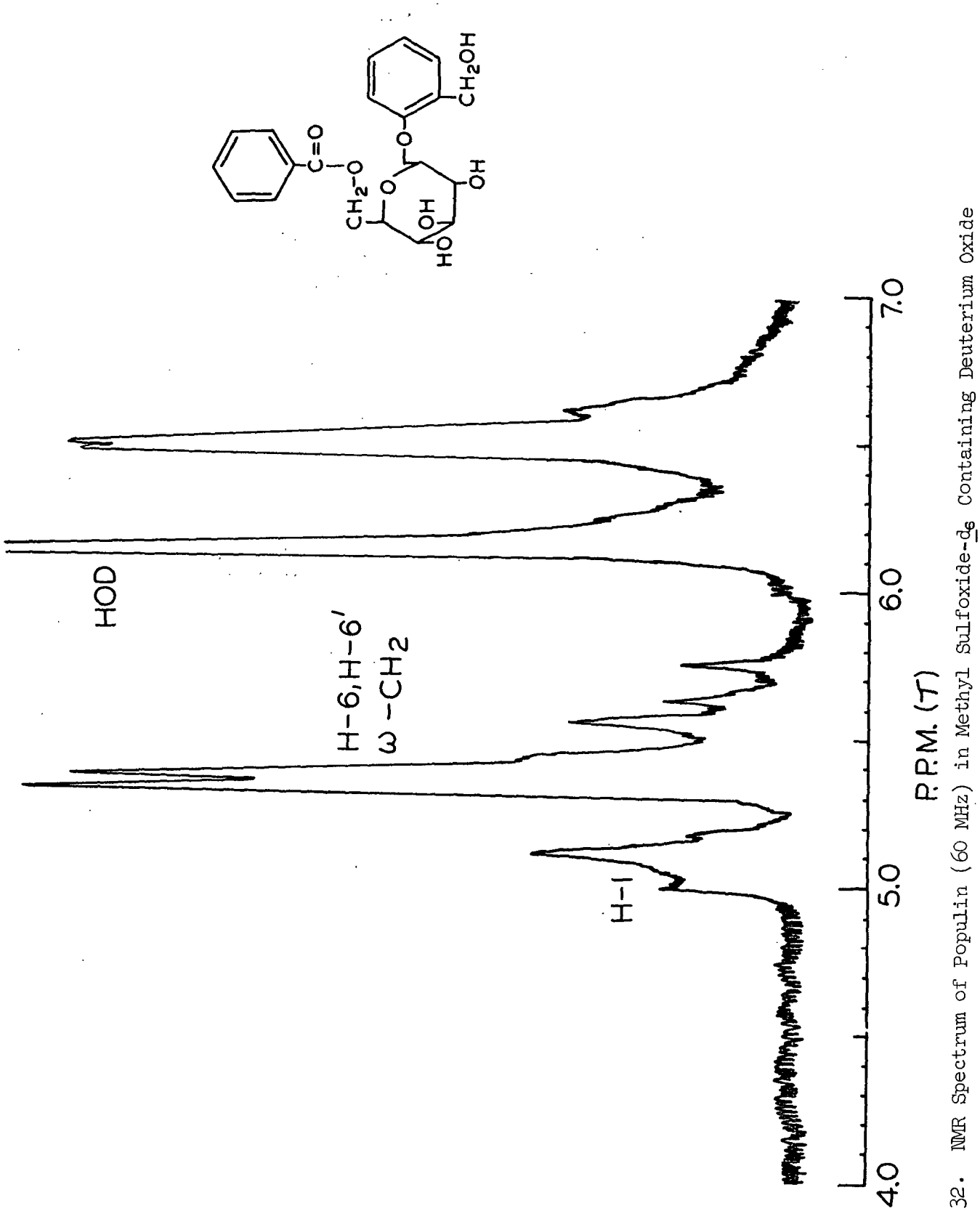

Figure 32. NMR Spectrum of Populin (60 MHz) in Methyl Sulfoxide-d_6 Containing Deuterium Oxide

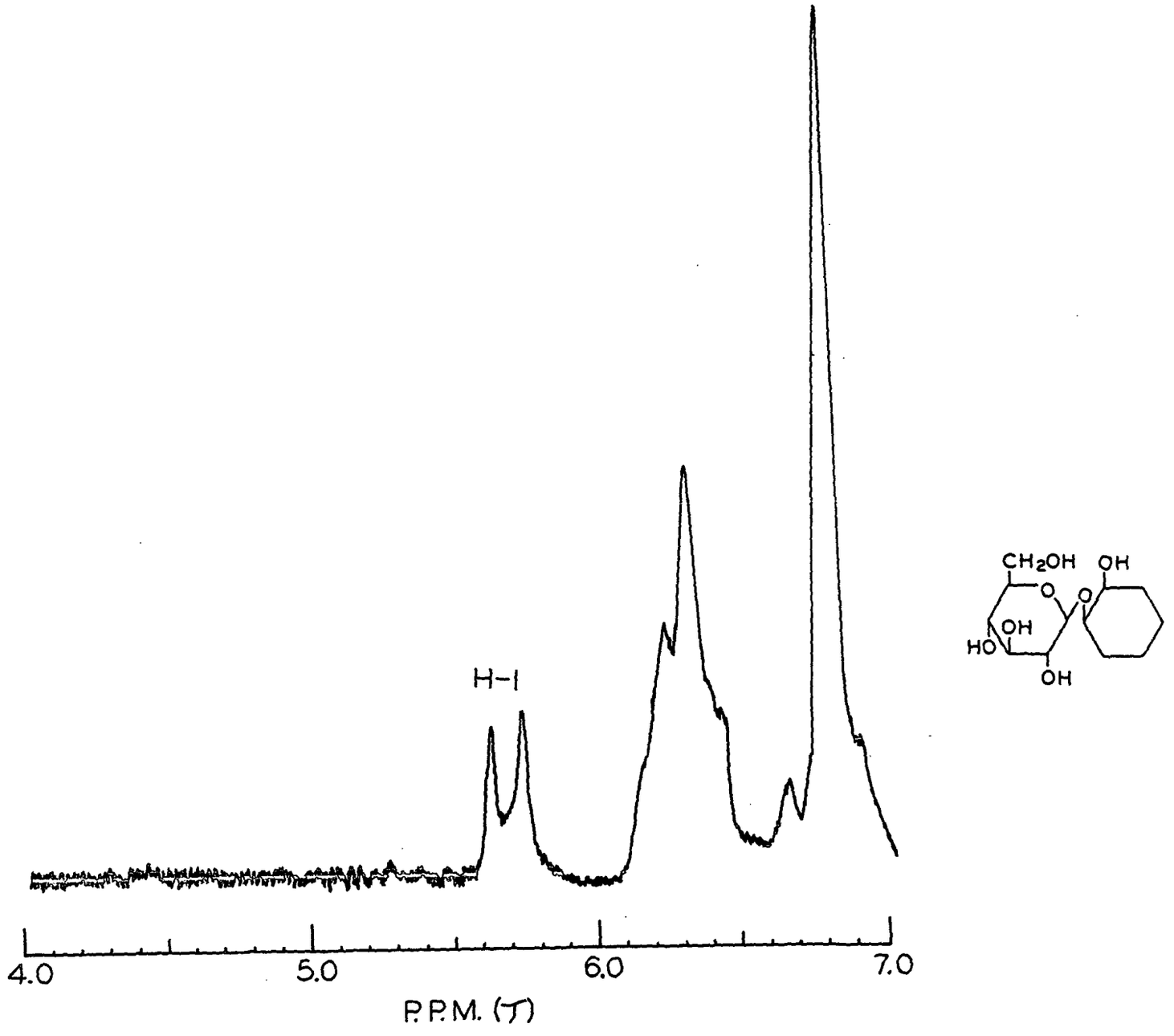

Figure 33. NMR Spectrum of Grandidentin (60 MHz) in Methyl Sulfoxide-d_6 Containing Deuterium Oxide and a Trace of Formic Acid

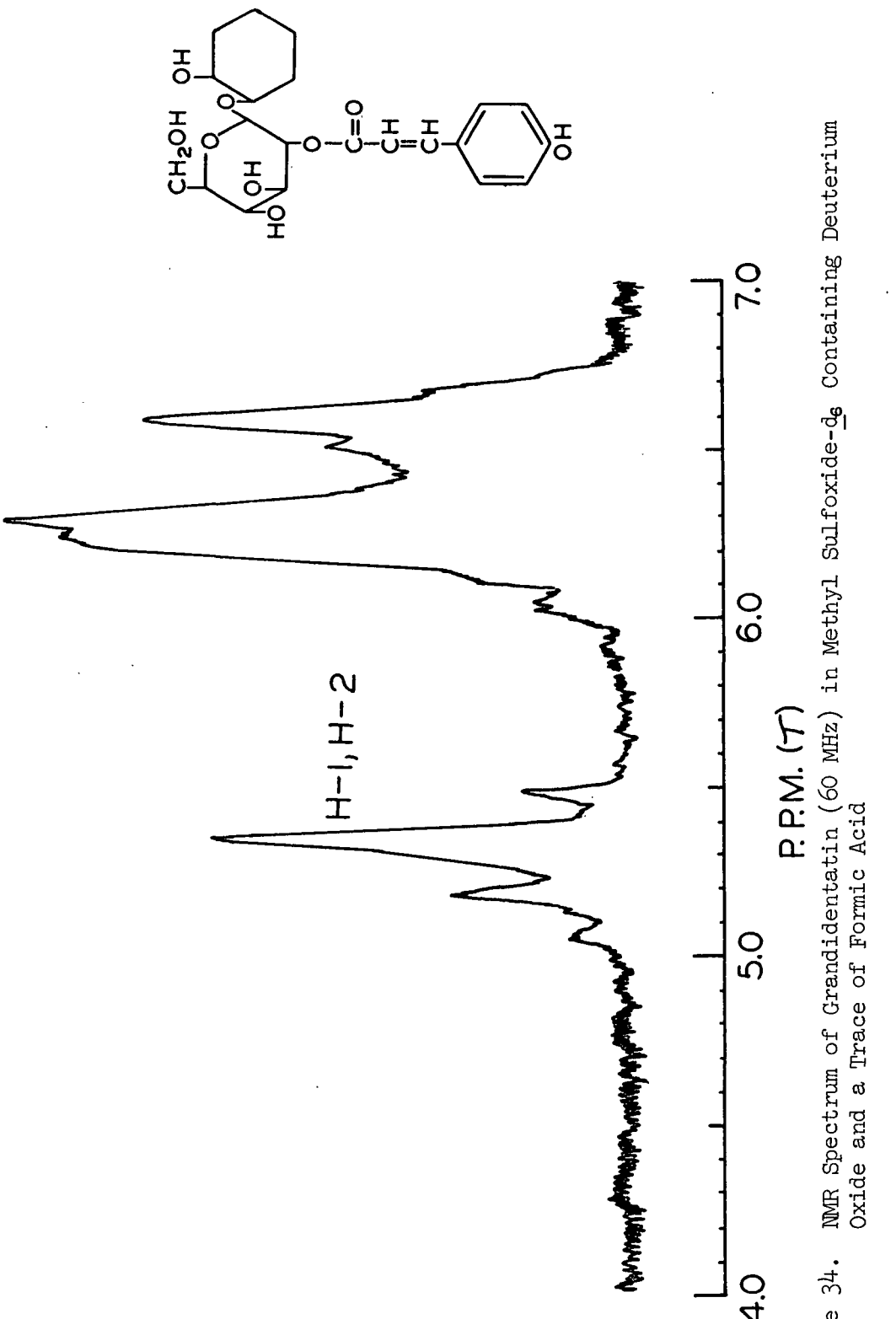

Figure 34. NMR Spectrum of Grandidentatin (60 MHz) in Methyl Sulfoxide-d_6 Containing Deuterium Oxide and a Trace of Formic Acid

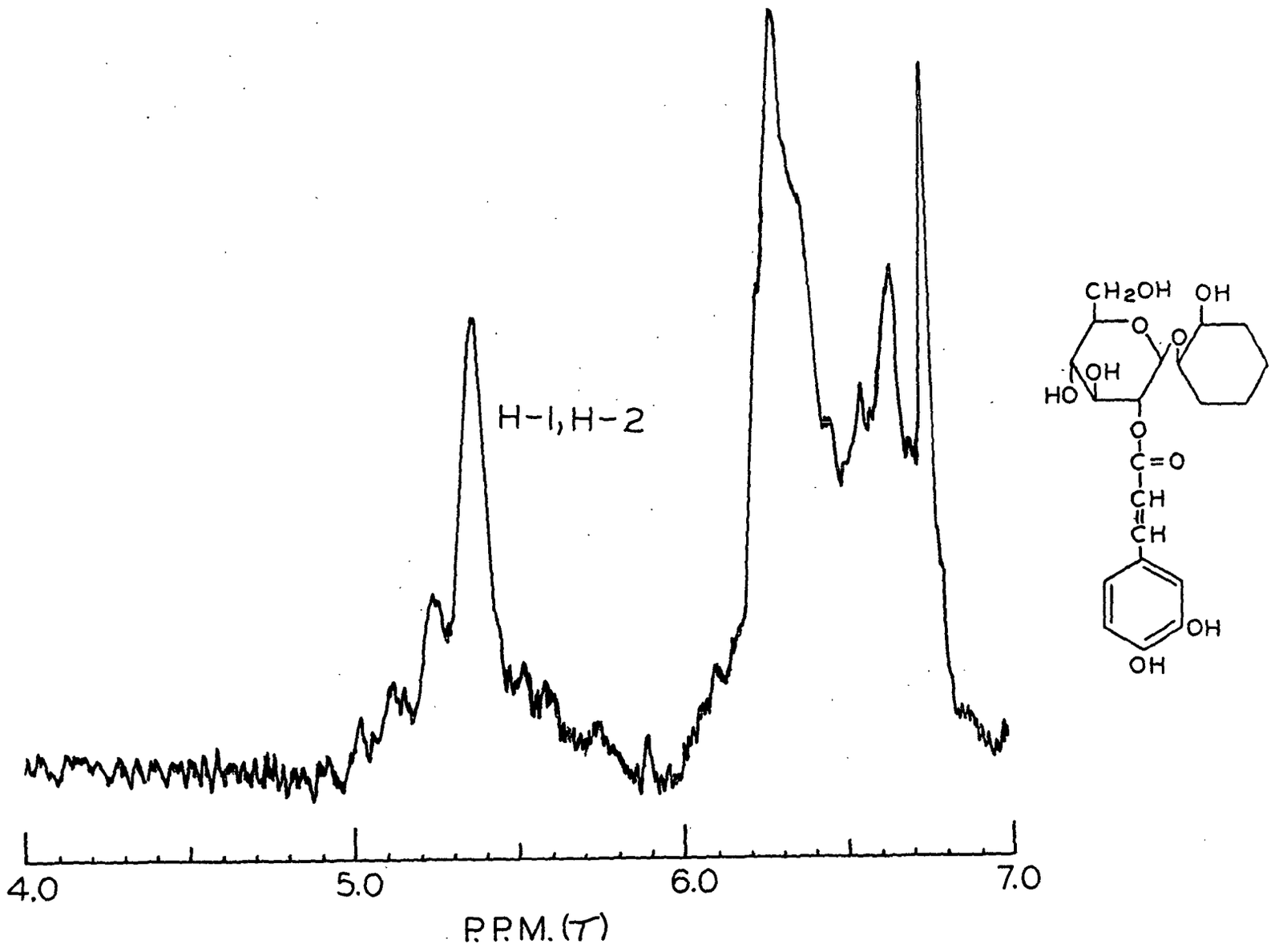

Figure 35. NMR Spectrum of Grandidentoside (60 MHz) in Methyl Sulfoxide-d_6 Containing Deuterium Oxide and a Trace of Formic Acid

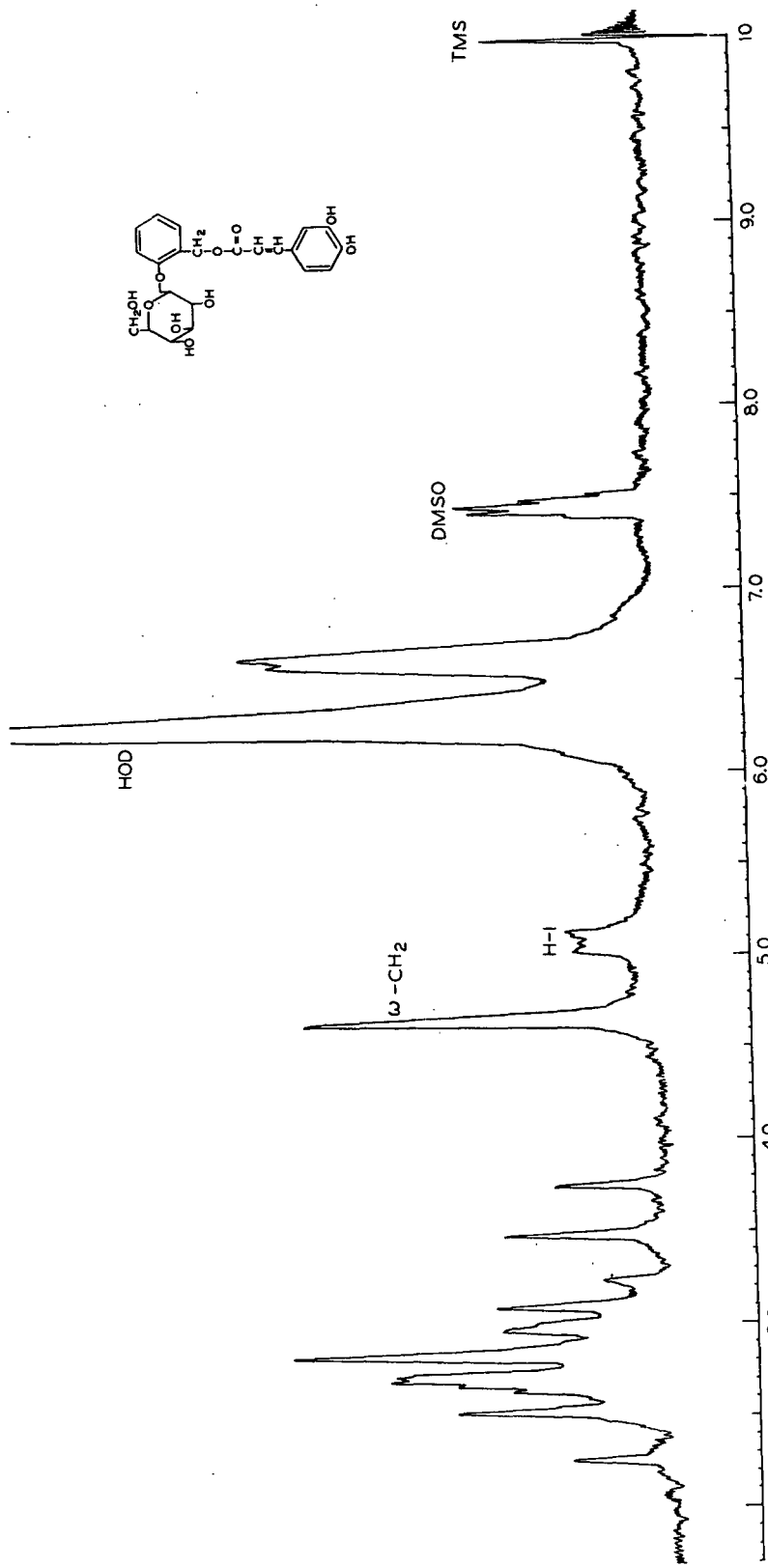

Figure 36. NMR Spectrum of Populoside (60 MHz) in Methyl Sulfoxide-d_6 Containing Deuterium Oxide

APPENDIX V

PROPERTIES OF KNOWN SUBSTANCES FROM THE FAMILY SALICACEAE

Compound	Melting Point, °C.	TLC R_f[a]	TLC Color[b]
1-*O*-*p*-Coumaroyl-β-D-glucose	205-60	0.31	Gray
Fragilin	177-9	0.50	Pink
Grandidentatin	202-4	0.44	Slate-green
Grandidentoside	196-7	0.35	Lavender
Picein	194-6	0.57	Tan
Populin	180-2	0.53	Pink
Populoside	186-8	0.32	Lavender
Pyrocatechol	105	0.78	Blue-gray
Salicin	200-1	0.30	Pink
Salicyl alcohol	86	0.80	Pink
Salicyloyl populin	180-1	0.85	Pink
Salicyloyl salicin	168-9	0.57	Pink
Salicyloyl tremuloidin	192-3	0.83	Pink
Salidroside	159-60	–	–
Salirepin	172-3	0.18	Brown
Salireposide	204-5	0.43	Yellow-brown
Tremuloidin	211-2	0.55	Pink
Triandrin	177-9	0.30	Blue-rose
Trichocarpin	134-6	0.45	Green-black
Trichocarposide	180-2	0.46	Weak gray-pink
Trichoside	163-5	–	Green-black
Vimalin	143-4	0.47	Blue-rose

[a] R_f in 4:1 chloroform-methanol (trace of acetic acid).

[b] Color after spraying with sulfuric acid and heating at 105°C. for 10 min.

APPENDIX VI

INFRARED ABSORPTION SPECTRA

Figures 37 - 40 are the infrared absorption spectra of populoside and grandidentoside and their corresponding acetates. Figures 41 and 42 compare grandidentin pentaacetate and caffeic acid, found during this study, with authentic samples of each component.

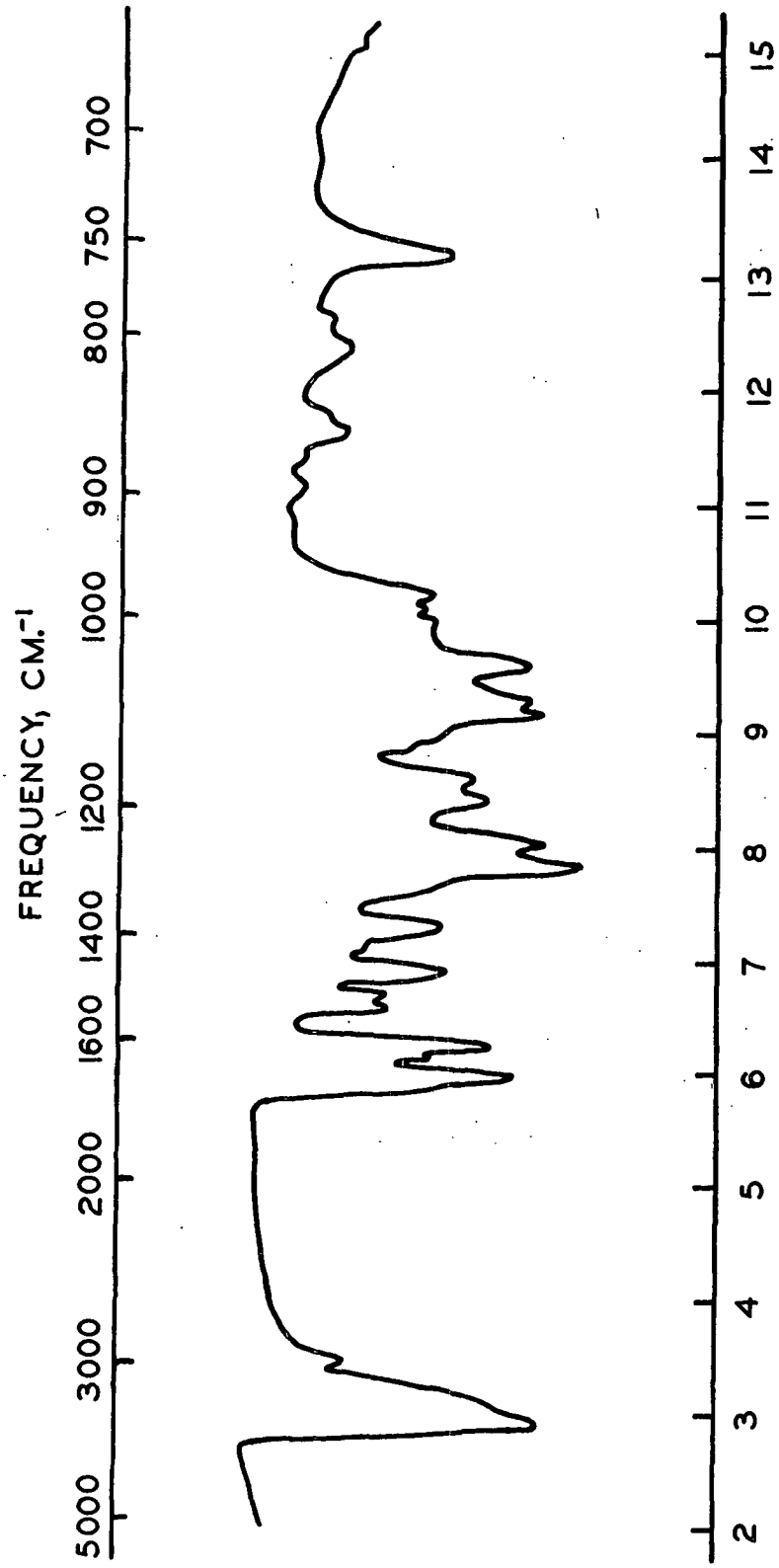

Figure 37. IR Spectrum of Populoside

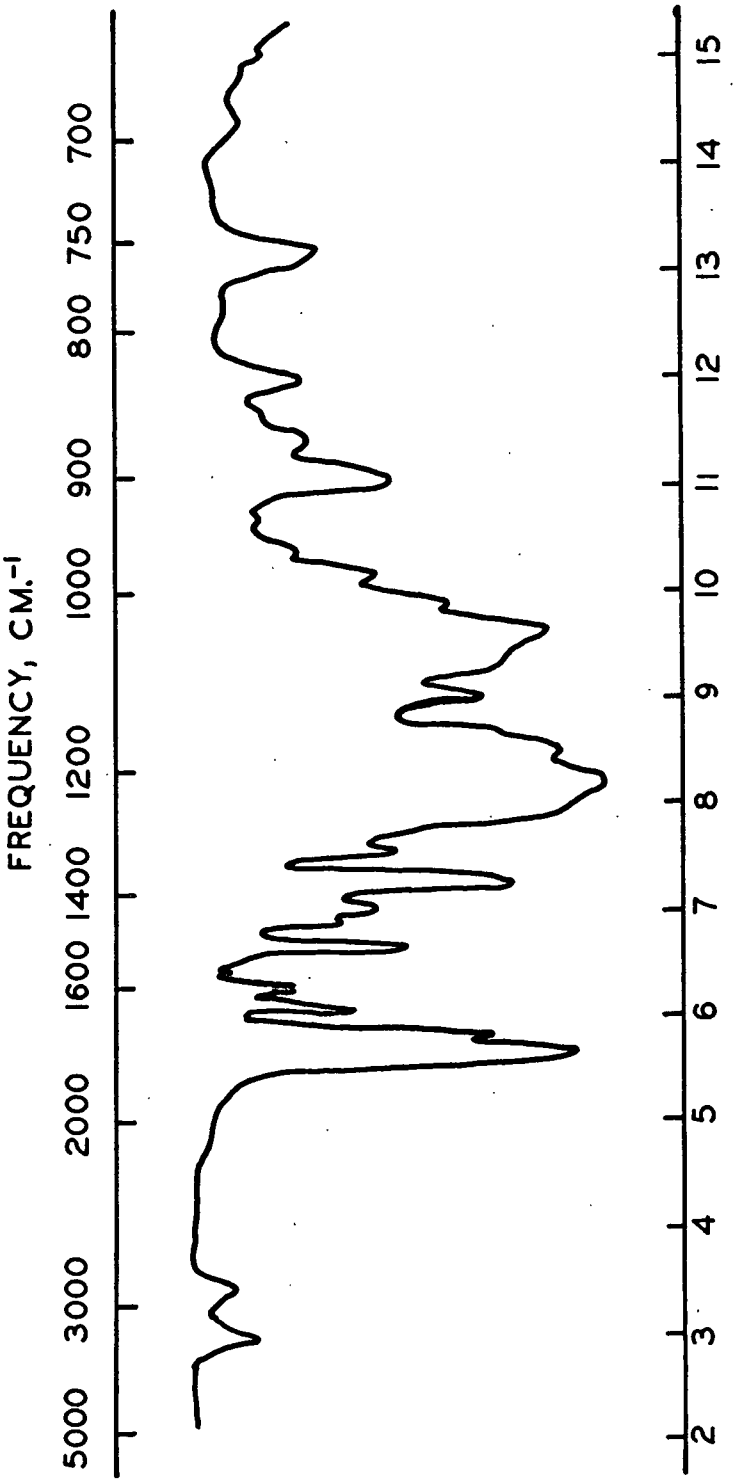

Figure 38. IR Spectrum of Populoside Hexaacetate

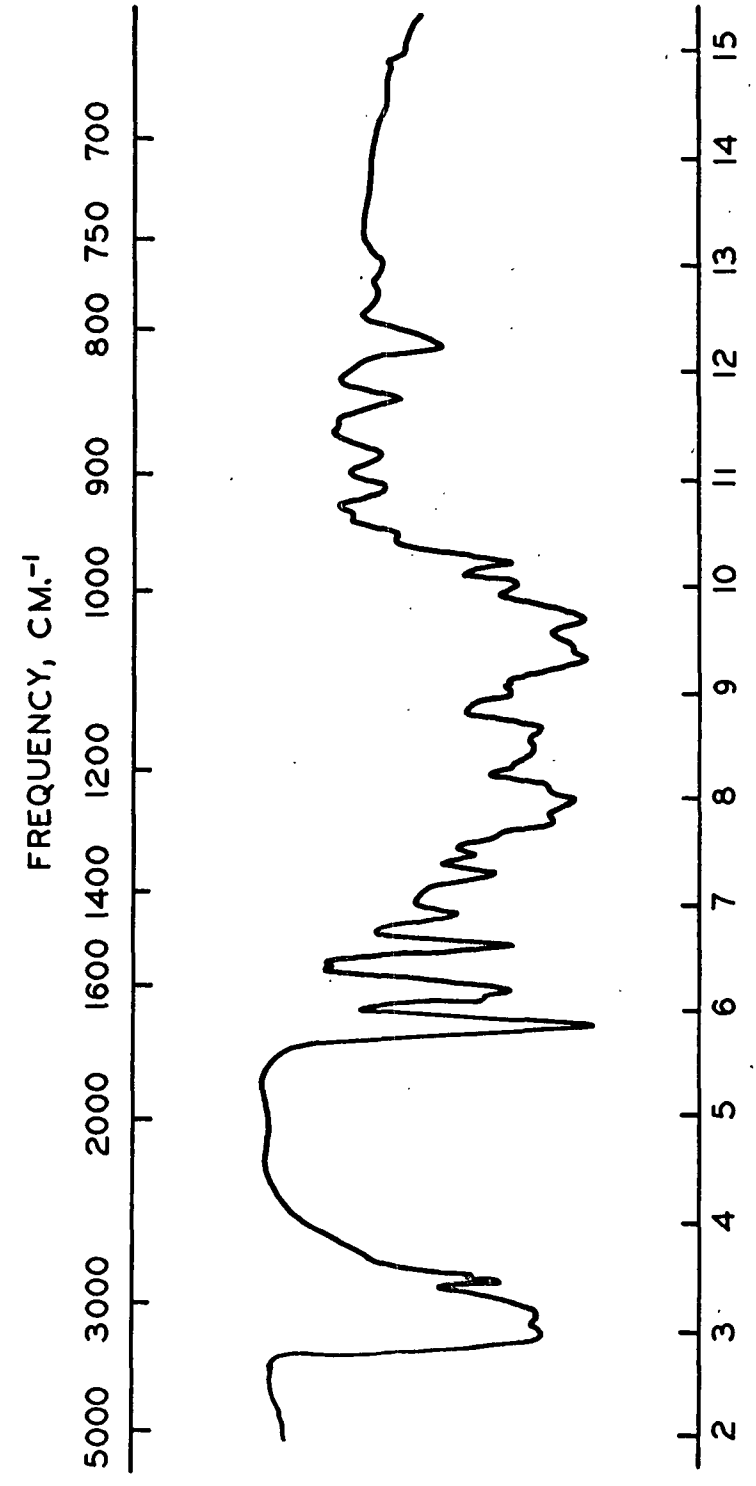

Figure 39. IR Spectrum of Grandidentoside

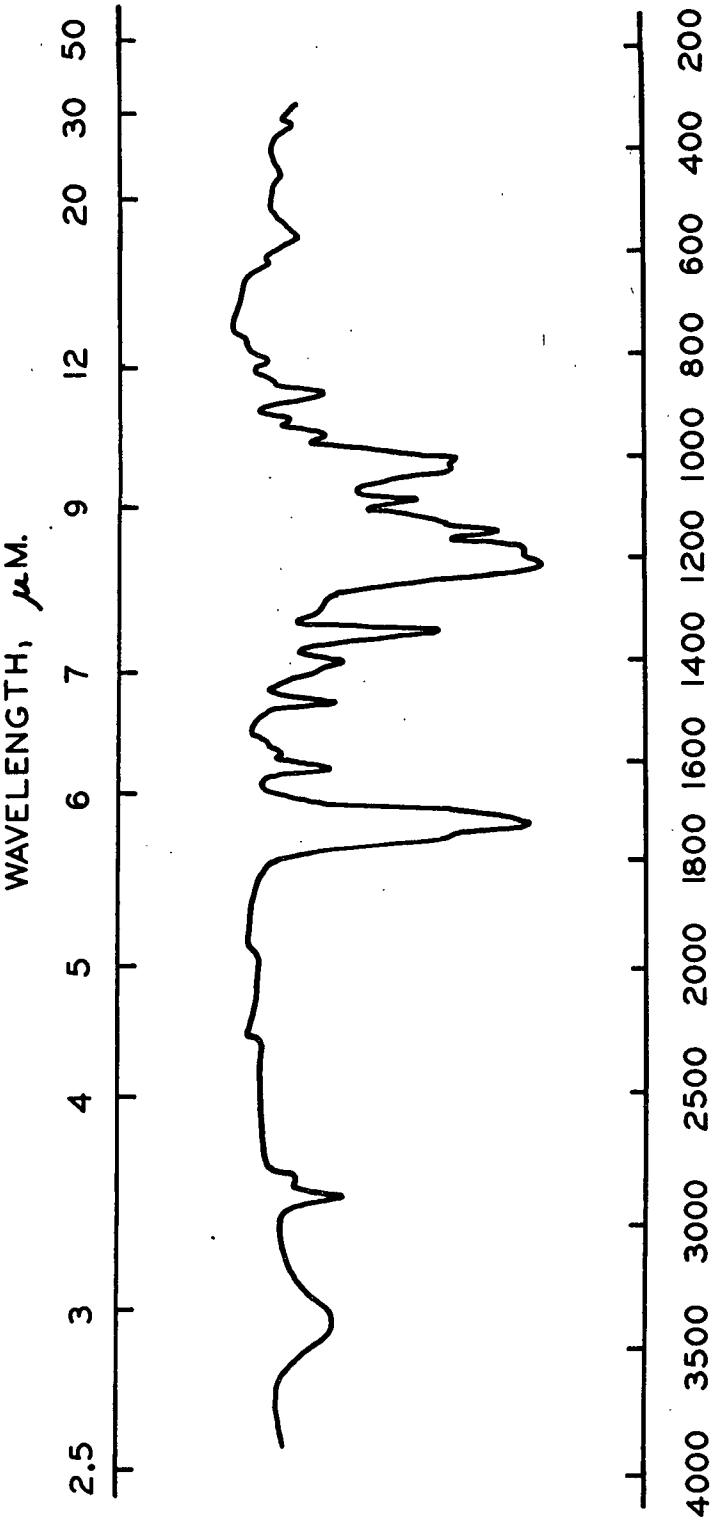

Figure 40. IR Spectrum of Grandidentoside Hexaacetate

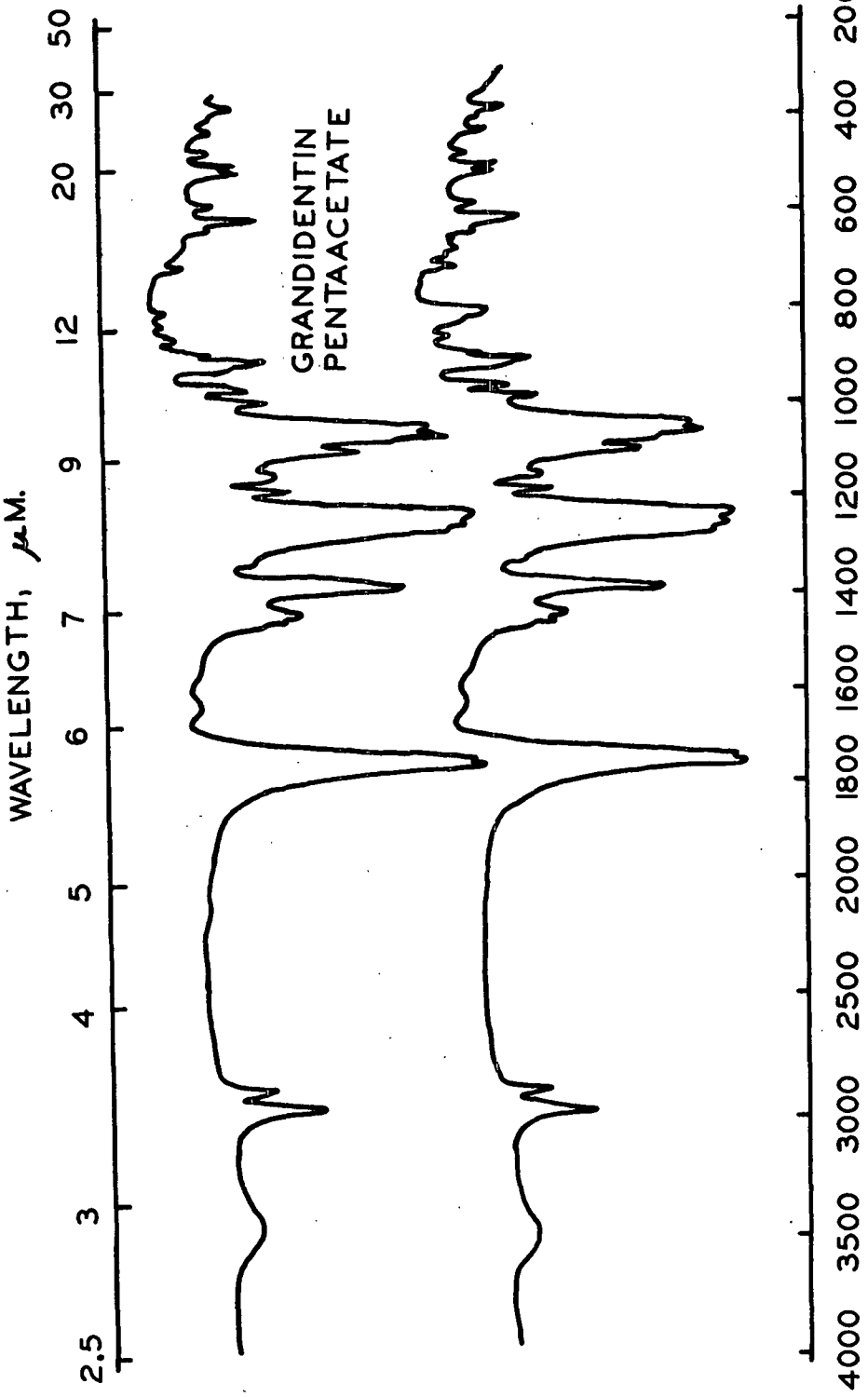

Figure 41. IR Spectra of Grandidentin Pentaacetate (Top) and the Decaffeoylated Grandidentoside Acetate (Bottom)

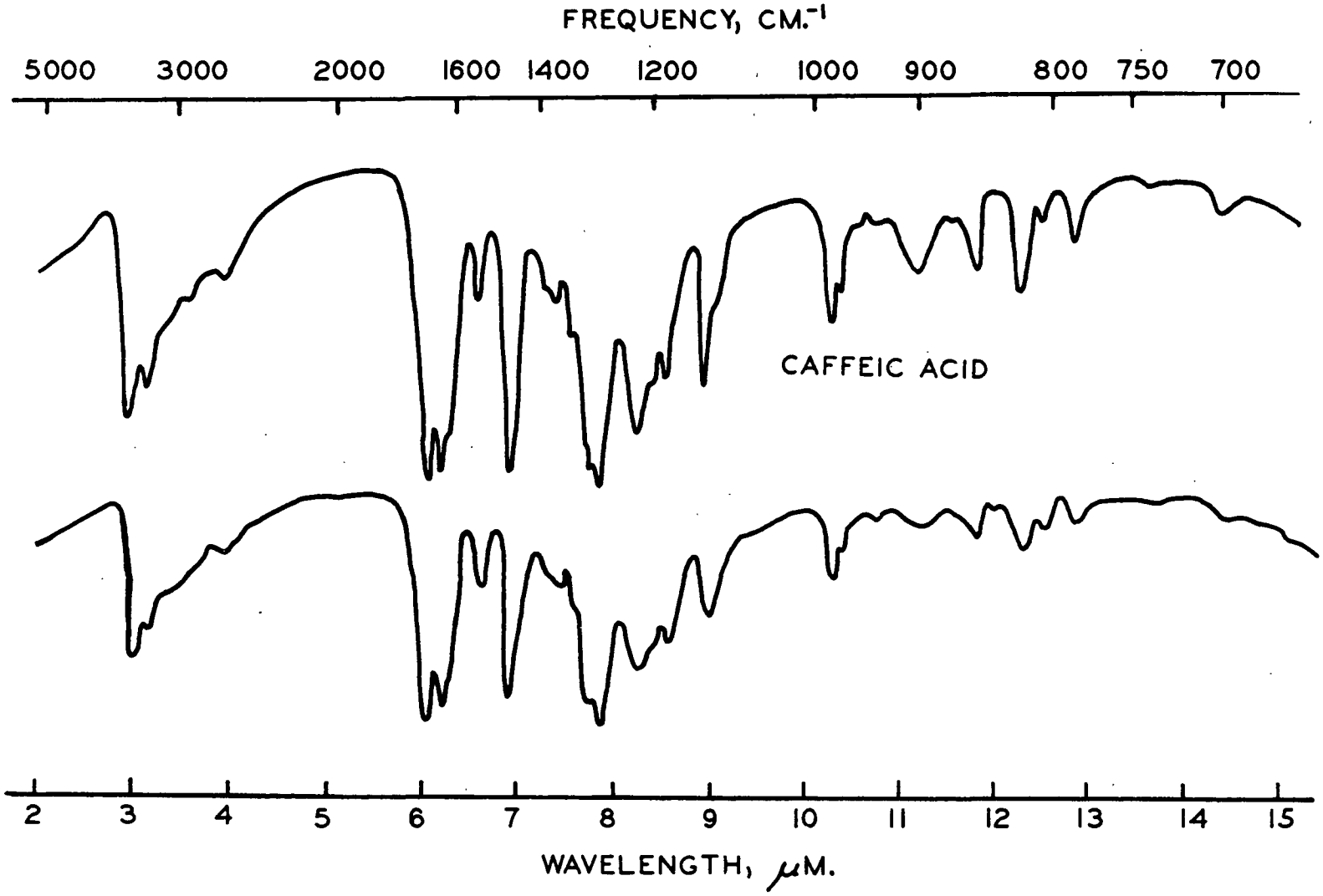

Figure 42. IR Spectra of Caffeic Acid (Top) and the Organic Acid from the Alkaline Saponification of Populoside (Bottom)